A STUDY OF AMYLOLYTIC STREPTOCOCCI

FROM THE RUMEN OF THE SHEEP

Thesis presented to the University
of Edinburgh for the degree of
Doctor of Philosophy

Margaret J. MacPherson

December 1953.

CONTENTS

INTRODUCTION

THE RUMINANT

Ruminants comprise a large class of mammals distributed over the greater part of the earth's surface. The digestive system of these animals has been adapted for the digestion of grass and other cellulosic materials which form the natural diet of the group as a whole. Since this diet is both bulky and indigestible it is to be expected that the ratio of the capacity of the digestive system to body weight is higher in ruminants than it is for example in carnivorous animals. For this purpose the lower part of the oesophagus in the ruminant forms a large sac or rumen. The ingested food passes into the rumen and is mixed with the dense rumen microbial population. It is in the rumen that the digestion of cellulose by rumen bacteria takes place. The relationship between the rumen microflora and the host is thought to be symbiotic and since a large percentage of the world's human population is dependent directly or indirectly upon ruminants for food the economic importance of this symbiotic relationship cannot be over emphasised. Especially as, but for these animals much of the yearly crop of

grass and other cellulosic material would not be converted into protein but broken down by soil bacteria and so be lost to man.

In most parts of the world the continuous growth of plants is not possible, due to the sequence of the seasons or to the alternation of wet and dry periods. Thus, at intervals, herbivores must live on vegetation which has completed its growth cycle and has become dry, woody and resistant to digestion. The digestive system of the ruminant can utilise such material and as a result these animals are not seriously affected by drought and other inclement weather conditions. This may account for the success of the group in the modern world. In addition it is this ability to utilise cellulose efficiently that has led to the domestication and breeding of the ruminant.

DIGESTION IN THE RUMINANT

The digestive system of the ruminant is specifically adapted for the digestion of large quantities of grass and other fibrous foods. Fig. 1 is a diagram showing the structure of the rumen. It shows that the rumen is a large diverticulum at the lower end of the oesophagus. Food swallowed usually passes directly into the rumen. It may however pass

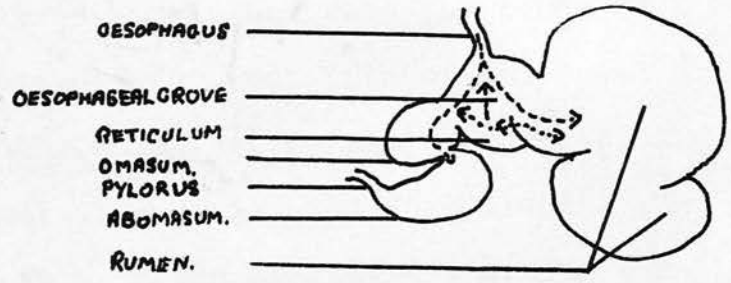

Fig. 1. Diagram of the upper part of the digestive tract of a ruminant.

from the oesophagus into the reticulum and then return to the rumen. In the young animal the rumen is only slightly developed and while the diet consists of milk the oesophageal groove enables food to by-pass the rudimentary rumen. However as roughage is introduced into the diet the rumen enlarges until in the adult sheep it is said that approximately one tenth of the total body weight is made up of food in the rumen. In the bovine this figure is even higher and when the rumen of a cow is full it accounts for one fifth of the total body weight of the animal (i.e. the capacity of the rumen of an animal of 1000 lbs. is 25 gallons). The total weight of food in the digestive tract of the ruminant is greater than it is in non-herbivorous species, this is probably because digestion is comparatively slow and several days may elapse between the ingestion of a meal and its subsequent elimination. A constant churning and mixing of the contents are maintained by muscular contractions of the rumen wall which take place once every minute. Under natural conditions the animal will graze for a period, filling the rumen with unchewed material. Grazing is followed by a period of rest when rumination or cud chewing takes place. In this

process the ingested material is circulated from the rumen into the reticulum and thence, probably by a sucking movement is brought up into the mouth. There it is thoroughly chewed and passed back into the reticulum, whence it may either pass back to the rumen or on to the omasum. The contents of the rumen are by these processes brought into a finer state of sub-division. The omasum has a number of skin-like flaps between which the fluid material leaving the rumen must flow. The filtering action of these flaps holds back large particles so that material passing on to the abomasum is quite fine. The abomasum is comparable in function to the stomach of non-ruminant animals and contains the same digestive secretions. The remaining portions of the gut are similar to those of non-herbivorous animals, the abomasum being followed by a small and large intestine.

The rumen has no associated digestive glands. This fact, coupled with the size of the rumen, led to the belief that its sole function was to act as a large storage chamber. Suggestions that the abundant bacteria in the rumen were beneficent symbionts rather than mere commensals were first made by Tappeiner (1882) and Zuntz (1891) who found

that the disappearance of cellulose in the alimentary tract of the ruminant could not be ascribed to any digestive enzymes secreted by the animals themselves.

The discovery that microbial enzymes played an important part in the digestion of the ruminant resulted in modifications of earlier theories about the digestive mechanism. Descriptions of digestive processes in higher animals have been traditionally based on knowledge of enzymes secreted by the body, since these enzymes can be separated and studied in vitro. Such enzymes bring about degradation of the food which is thus enabled to pass through the wall of the intestine. If it is the case that some of the enzymes effecting these changes are derived from micro-organisms growing in the alimentary tract and not from the digestive glands of the host, it still seems proper to consider such enzymes as part of the digestive mechanism. An amylase of microbial or plant origin which is active in the alimentary tract, ought therefore, to be regarded as a digestive enzyme.

The synthesis of new material by bacteria, which may take place in the rumen is, on the other hand, a different matter and it is questionable

whether it should be included under the term "digestion" any more than are other synthetic processes going on outside the alimentary tract. It would seem, therefore, desirable to differentiate between processes of degradation and those of synthesis when considering metabolic processes in the rumen.

The contents of the rumen.

The contents of the rumen may be considered as comprising three phases :-

1. The solid phase.
2. The liquid phase.
3. The gas phase.

The solid phase.

This is made up of two components (a) the food (b) the microbial population. The food of a ruminant under natural conditions consists largely of grass and other herbage and a sheep consumes 1 kilogram of herbage per 100 lbs. body weight per day. The total carbohydrate intake is, therefore, high and consists largely of cellulose and soluble sugars. The latter fluctuate throughout the year, being high in the spring and low at the end of the growing season. Hale, Duncan and Huffman (1947) showed that, in the ruminant, 80% of the cellulose

utilised was digested in the rumen. Gray (1947) obtained the slightly lower figure of 70 - 75%. Under artificial conditions supplements are added to the diet in the form of cereal starches and protein, these enable the animals to put on weight more quickly.

The microbial population of the rumen includes both protozoa and bacteria. The protozoa were first described by Gruby and Delaford (1843) who, although they did not provide illustrations of their findings, recognised several distinct types. They postulated that, since a large number of protozoa were found in the rumen, they must be an important functional part of the rumen flora. Mangold (1920) described the protozoa found in the rumens of several animals and recognised that the species varied in number and proportion according to the nature of the diet. Entodinium, Diplodinium, Isotricha and Butchlia were the species most common in the rumen (Van der Wath and Myburgh, 1941; Baker, 1942; Hungate, 1946).

Mowry and Becker (1930) showed that, in goats, the numbers of protozoa increased as the amount of starch in the diet was raised. Van der Wath and Myburgh (1941), using the counting technique of Van der Wath (1948), gave the numbers of protozoa in

sheep fed on diets of lucerne-hay and wheat straw and of lucerne and maize meal as about 1000/cu. mm. and 3,500/cu.mm. respectively. Maize alone caused a marked reduction in numbers of protozoa which these workers thought was due to an increase in the quantity of fatty acids in the rumen. This explanation is open to doubt, since rumen protozoa normally exist in an environment containing fairly large quantities of fatty acids. It would seem that an increase in acidity due to inadequate buffering of the acids, is a more reasonable explanation for the decrease found in the numbers of the protozoa in sheep on high maize diets. Van der Wath and Myburgh did not find any difference in the numbers of protozoa present in sheep on dry and on green lucerne rations. This was contrary to the findings of Trier (1920) who found larger numbers with the green lucerne diet and suggested that the rise was due to the higher chlorophyll content. Oxford (1951), who observed a similar increase, attributed it rather to the higher content of soluble sugars. This seems a much more likely explanation since protozoa have been shown to ferment sugars rapidly and so might be expected to increase when the latter are abundant.

Johnson, Hamilton, Robinson and Garey (1944) destroyed all the protozoa in the rumen by the administration of copper sulphate ("defaunation") and observed that the animals continued to thrive and to digest cellulose as before. Hungate (1946), however, has suggested that one cannot deny functions to the protozoa in the economy of the rumen on such purely negative evidence. It might, for example, mean that the removal of protozoa from the rumen has permitted an increase in a part of the bacterial flora so that the end result in terms of cellulose digestion is the same. While, therefore, the protozoa in the rumen have not been shown to be essential to the well-being of the host, their presence in such large numbers makes it probable that they exert a considerable effect upon processes normally taking place and their possible importance cannot be ignored.

Tappeiner (1882, 1884) was the first to establish the importance of bacteria in the decomposition of food in the ruminant. He demonstrated the digestion of cellulose by rumen bacteria by incubating filter paper in broth inoculated with rumen bacteria. By this method he showed that some 35% of the filter paper could be

digested. He also proved that these changes were not effected by cell free enzymes. Pringsheim (1912) was also unable to demonstrate an extra-cellular cellulose in such preparations and he stated that for the microbial breakdown of a complex substrate like cellulose, intimate contact of the organism with the substrate was necessary. His work was later confirmed microscopically by Henneberg (1922) who showed that, in the rumen, the cellulose components of the food undergoing decomposition were surrounded by micro-organisms which stained blue with iodine and which produced cavities in the cellulose fibres. He believed that these organisms were responsible for the breakdown of the cellulose. Although his view was criticised by Thaysen and Bunker (1927), it has been amply confirmed by the work of Baker and Nasr (1947). The latter showed that all carbohydrate materials undergoing digestion in the rumen or caecum of herbivorous animals were surrounded by large numbers of cocci and bacilli which stained blue with iodine. Baker and Martin (1938) observed that in the caecum of the horse the micro-organisms attacked pectic substances as well as carbohydrates. These workers also found that heavily lignified structures were

not attacked, this is in keeping with the low digestibility of lignified plant tissues.

Provided that the diet is constant in composition, the organisms attacking the plant particles are of fairly constant morphological types (Moir and Masson, 1952). Baker (1942) described iodophillic organisms including Oscillospira guillermondi, a giant spirillum, a sarcina and a rosette-shaped organism which he called asteroides. Baker and Harris (1947) described curved rods, vibrios, very large and very small coccoids, an ellipsoidal organism and other elongated forms, all of which they believed to be associated with cellulose digestion.

Gall, Burroughs, Gerlaugh and Edgington (1949) and Bortree, Smith, Sakar and Huffman (1948) examined the effects of different rations on the micro-organisms attached to the solid portions of the food and found that the population was morphologically relatively constant. The organisms not attached to food particles, however, were found to vary with the diet. Gall et al. found asteroides in the rumen contents of pasture fed animals but not in animals which had been stall fed. Similarly, Moir (1951) found oscillospira in rumen contents of

animals at pasture but not in those receiving a hay diet.

The addition of methionine to the diet caused the appearance of a giant spirillum in the rumen (Bortree et al. 1948). Increases in the soluble sugar content resulted in an increase in the numbers of protozoa and also in the appearance of an oval-shaped organism (Quin, 1943; Elsden, 1945). This organism metabolises glucose rapidly and has been shown to be connected with the fermentation of sugars in the diet (van der Westhuizen, Oxford, and Quin, 1950). The introduction of glucose into the diet of a sheep on poor quality hay led to a rapid fermentation and the accumulation of lactic acid, whereas glucose given to animals on a better diet of pasture or good quality hay was rapidly and completely metabolised (Quin, 1943, Elsden, 1945).

Van der Wath (1948) found that starch in the rumen was attacked by a flora of cocci. This micro-flora became very highly developed if starch was continuously present in the diet. The rapidity of digestion of starch grains varied with different starches. He carried out experiments with sheep, introducing raw starch through a rumen fistula. Microscopically he observed that maize starch was

completely digested in some 18 hours. This time was, however, increased to about 24 hours if the previous diet had not contained any starch supplement. Potato starch, on the other hand, remained undigested and intact granules could still be found in the rumen even after 28 hours after feeding. Van der Wath also correlated the speed of digestion with the size of the grain. However Baker, Nasr, Morrice and Bruce (1951) thought that this was much more dependent on the surface of the grain. If the grain was intact, digestion was much slower than if the grain was first damaged by grinding in a ball-mill.

It would seem that the bacterial population of the rumen can be provisionally regarded as being made up of some species which are constantly present and others whose presence is dependent on the diet. This conclusion is not unexpected since in any mixed culture the relative numbers of the various species will depend on the ease with which they can utilise the nutritive substances which are available.

The liquid phase.

The liquid in the rumen consists of saliva diluted with ingested water and plant juices. Its composition varies with the diet but since the

saliva forms the major component, variations are not so great as might be expected.

McDougall (1948) made a complete analysis of sheep saliva. He found that sheep saliva contained about 1% of solids and had an ash content almost identical with the total solids, showing that little organic matter was present. The carbon dioxide content was about 270 volumes per cent and the phosphate 80 mg. per 100 ml. The former was four times and the latter twenty times the corresponding values for blood, showing that the saliva was a true secretion (cf. Watson, 1933). Levels of Na and K, on the other hand, were similar to those in serum.

The volume of saliva secreted by the sheep was estimated by Mc Dougall to be from 2 - 3.5 litres/day (cf. Watson's (1933) figure of 4.5 litres). The saliva had a $_{p}H$ of about 8 and, with its high content of bicarbonate, McDougall estimated that the amount secreted per day was adequate to buffer effectively the fermentative production of fatty acids in the rumen liquor. It was until recently thought that the rumen contents were alkaline. Pochon (1933) gave the $_{p}H$ as 8.0. This figure is now thought to be too high since it was obtained from rumen contents of slaughtered animals (which had

been starved for considerable periods) and no precautions were taken to avoid loss of CO_2 before measurements were made. Smith (1941) measured the $_pH$ in the rumen by electrodes introduced through a fistula and found a value of 6.3 for cattle fed on alfalfa hay and a lower value of 5.5 for cattle fed on hay and a beet pulp. Marston (1945) recorded values of 6.0 - 6.4. It is thus clear that the rumen has a $_pH$ slightly on the acid side of neutrality.

In the rumen, as in other organs of the body, a constant internal environment is maintained. The temperature is constant (39°C) showing only slight species variation, this in itself helps to maintain a constant type of microbial population. Since fatty acids are one of the main products of the breakdown of carbohydrates in the rumen, the $_pH$ of rumen contents tends to fall and without adequate buffering this would inhibit the growth of the rumen bacteria. The large volume of alkaline saliva, however, is sufficient to prevent the acidity from becoming too high. The feeding of acid foods like silage increase the flow of saliva to compensate for any lowering of the $_pH$ which might take place.

The non-mineral components of the saliva,

though present only in small amount, have a large effect on the wetting properties of the saliva. The average nitrogen content of sheep saliva is 20 mg.% (McDougall, loc. cit.), contained mainly in mucoid proteins. Ried and Huffman (1949) showed that bovine saliva had a low surface tension (47.1 dynes/cm.^{2}) compared with that of water (75.3 dynes/cm.^{2}). It is possible that the bacteria in the rumen are favoured by this low surface tension which may act selectively by preventing air and soil bacteria from establishing themselves in the rumen. Due to the surface effects of the large quantity of saliva secreted by the ruminant, chemical reactions and bacterial processes dependent on solution, wetting, and mixing are facilitated.

The saliva, finally, contains about 1.5 mg. per litre of ascorbic acid. It has been suggested that this constituent may help to regulate the oxido-reduction potential. However, the true significance of this constituent is still uncertain.

Data on analyses of rumen liquid itself are scantier than those available for saliva. Its nitrogen content is low. In sheep on meadow-hay McDougall (1948) found 45 - 75 mg.% protein nitrogen and 23-66 mg.% non-protein nitrogen. 17 - 43 mg.% of the latter was ammonia nitrogen. Rumen liquid also contains vitamins of the B-complex (thiamine,

biotin, riboflavin, nicotinic acid, pantothenic acid and pyridoxin). Vitamin K has also been shown to be present (Smith, 1945, Kon and Porter, 1947). In addition to these, it seems that other as yet unknown factors are present, since rumen liquor was found to be essential for the growth of certain organisms (some of which were cellulose decomposes) isolated from the rumen (Bryant, 1952, Sjipesteijn, 1948, Hungate, 1950). These factors could not be replaced by the addition of yeast extract.

Garton (1951) estimated the amount of inorganic phosphorus, soluble magnesium and soluble calcium in the rumen of the sheep and found that the soluble inorganic phosphate in rumen liquid varied quite surprisingly with the diet. McDonald (1952) also found that the concentration of the various forms of non-protein nitrogen, including NH_4 in rumen liquid, varied both with diet and the time after feeding at which the specimen was obtained.

Further knowledge of the composition of rumen liquid is clearly desirable, since it determines the environment in which the rumen micro-organisms grow. In many of the attempts to isolate rumen organisms, this self-evident fact has been ignored and the media used have borne little similarity to rumen

liquid.

The gas phase.

Carbon dioxide and methane are the predominant gases in the normal rumen, together with a variable percentage of nitrogen (3 - 30%) and oxygen (0 - 7%) which are probably swallowed as air (Washburn and Brody, 1937, Cole and Kleiber, 1948). Pilgrim (1948) found that the proportions of carbon dioxide and methane fluctuated during the feeding cycle. Both were rapidly evolved during the first few hours after feeding, carbon dioxide to a greater degree than methane. This was particularly so if the feed contained large amounts of soluble sugars.

Although Pilgrim found no hydrogen in the rumen gases of sheep fed on chopped hay, he observed, in fistulated sheep, that following starvation or removal of the rumen contents through the fistula, hydrogen was formed on re-feeding. Production of hydrogen reached its maximum after 12 - 24 hours and was sometimes as much as sixteen times greater than methane production. Methane production could, however, be restored to normal levels and hydrogen production diminished by the inoculation of rumen contents from a normal sheep. From these observations Pilgrim suggested that two species of

bacteria were involved in the production of methane, one of them producing the hydrogen and the other using the hydrogen to reduce carbon dioxide to methane.

Tóth (1934) isolated nitrogen-fixing bacteria from the faeces of sheep and cows but failed to show either that these organisms could function anaerobically or that they were active within the digestive tract.

The Significance of Bacterial Protein in the Nutrition of the Ruminant.

Müller (1906) fed protein from rumen bacteria to dogs and showed that its nutritional value was similar to that of a ration of casein and albumen. Usuelli and Fiorni (1938) fed preparations made from protozoa and from bacteria to chickens, as supplements to a basal diet. They observed growth rate increases of 71% and 57% respectively, suggesting that the protozoal protein was more digestible than that from bacteria. Similarly, Johnson, Hamilton, Robinson and Garey (1944) fed preparations from protozoa, from a pure bacterial culture and from mixed bacteria to rats. The first and second of the preparations had digestibilities of 86% and 82% respectively, but that of the third

was only 55%. This low figure may, however, be due in part to impurities in the mixed bacterial preparation not present in that from the pure culture.

Smith and Baker (1946) by fractional centrifugation of rumen contents, obtained a bacterial preparation which was 36% protein and 47% starch-like polysaccharide. Similar results were obtained by McNaughton, Smith, Henry and Kon (1950) who found that the digestibility of the protein was low (73%) but its biological value was high (88%).

Thus it appears from the above evidence that the potential nutritive value to the ruminant of bacterial protein is high.

Estimates of the quantity of bacterial protein which is available for the host have varied widely. Kohler (1940) by direct counts in cattle estimated that only 23 g. of bacterial protein per day were available. These experiments were however carried out on material from poorly-fed slaughtered animals. Schwarz (1925) estimated that 12% of the protein in the rumen of slaughtered cattle was bacterial and Mangold and Schmidt-Krahmer (1927) suggested 10% for sheep.

Mills, Booth, Bohstedt and Hart (1942) made

the important observation that, when urea was fed together with starch, the protein concentration in the rumen was high. Urea and casein, on the other hand, gave a protein content no higher than casein alone. They concluded that the presence of starch was necessary for the utilisation of non-protein nitrogen for protein synthesis and that this process became more significant when the dietary protein intake was low. Pearson and Smith (1943) incubated rumen contents, to which urea was added, at 39° for 2 - 4 hrs. and measured the protein synthesised. They calculated that 25% of the total protein requirement of a cow yielding 25 lbs. of milk a day could be synthesised from urea.

Other <u>in vitro</u> experiments by Pearson and Smith led to a calculated value of 300 g. protein made available per day, provided synthesis proceeded at the same rate throughout the day. This last assumption is, however, unlikely in the case of animals fed only twice a day. Protein synthesis is here only likely to be maximal for some 4 hours after feeding and the amount available for the animal would then only be some 100 g. per day.

Hastings (1944) suggested that all of the protein in the diet of a ruminant was converted into

bacterial protein before it was absorbed. It seems likely, however, that an intake of as much as 1 kg. protein per day would all be converted into bacterial protein during its passage through the rumen.

Oxford (personal communication) has emphasised that although it is important to know that the protein of rumen bacteria has a high biological value, there is another aspect which must be considered, namely that protein of good biological value in food may, in certain circumstances be destroyed by rumen bacteria without any compensating advantage. Indeed the animal may be put to the added strain of eliminating the resulting ammonia as urea in its urine. This loss of good protein N. as NH_3 is far more serious than loss of C as CH_4 and may well be a potential source of weakness in the rumen metabolism as a whole.

The conclusion to be drawn from the observations quoted above seems to be that the total transformation of nitrogen (from all sources) into bacterial protein which is subsequently utilised by the host may well approximate to the host's total daily requirements. The actual amount of bacterial protein available lies between the extreme estimates of Kohler on the one hand and Hastings on the other. The possible loss of protein as a result of bacterial metabolism should also be considered.

MICRO-ORGANISMS ISOLATED FROM THE RUMEN

Only within the past 10 years has it been possible to isolate organisms in pure culture which can be said to be of some significance in the metabolism of the rumen. Most of this work has been concerned with cellulose fermenting organisms. The early work in this field was extensively reviewed by Sjipesteijn (1948) and Hungate (1950) who pointed out that all cellulose decomposing organisms previously isolated were spore-formers which had been isolated after prolonged enrichment culture or at temperatures which encouraged the growth of thermophilic organisms (Woodman and Stewart, 1928 and 1932). Furthermore, since conditions within the rumen with regard to pH, osmotic pressure and the composition of nutrient materials were not accurately understood, it was difficult for early workers to simulate the rumen conditions *in vitro*. For practically all of the early isolations simple salt media containing filter paper were used and conditions *in vitro* and *in vivo* were very different. It is, therefore, difficult to attach much importance to organisms isolated under conditions totally different from those encountered in the rumen. Until recently it was thought that

the $_{p}$H of the rumen was alkaline.

Almost all of the early work concerned with the detailed study of individual rumen organisms in pure-culture was done on cellulose fermenting bacteria. Pochon (1936) isolated Plectridium cellulolyticum from the rumen of the ox, sheep and other ruminants. He found that many related species were also present which showed a gradation in their growth requirements: those at one extreme had requirements similar to soil micro-organisms while those at the other extreme required anaerobic conditions and supplements of rumen contents in the medium. Unfortunately these cultures isolated by Pochon were grown at $_{p}$H 8.0 and would not grow at a $_{p}$H below $_{p}$H 7.0. Their significance in the rumen is therefore doubtful.

The major contribution to the present knowledge of the protozoa and the anaerobic and facultative anaerobic, mesophilic, cellulolytic bacteria of the rumen has been made by Hungate and Sjipesteijn. In 1942 Hungate isolated a protozon, Eudiplodinium neglectum, from the rumen of an ox and showed that this organism could ferment cellulose under anaerobic conditions. Cell free extracts of the cultures broke down cellulose to form glucose. He was unable to isolate bacteria from these cell free

extracts and, therefore, concluded that the cellulose was degraded by protozoal enzymes and not by those of symbiotic bacteria. This point is of interest when comparing the protozoa and bacteria in the rumen since Van der Wath and Myburgh (1941) found that Diplodinia predominated in the rumen of animals fed on a diet rich in cellulose. From this it seems logical to assume that the protozoa are important agents in the digestion of cellulose in the rumen. Hungate (1943) isolated three other Diplodinia and an Entodinium from the ox rumen. One culture of a Diplodinium examined could utilise cellulose. These cultures, when starved and placed on cellulose, deposited an iodine staining carbohydrate in their cells within one hour of their addition to the substrate. The Entodinium did not use cellulose but ingested considerable quantities of starch. This finding was also confirmed by Van der Wath and Myburgh's (1941) observation that, with a starch rich diet, Entodinium predominated. Hungate divided the protozoa into two groups

(a) Symbionts i.e. Diplodinia, which utilised cellulose.

(b) Non-symbionts or commensals i.e. the genera Dasytricha, Butchlia, Entodinium and Isotricha.

Turning to the isolation of pure cultures of bacteria from the rumen, Hungate (1946) isolated several strains of a Gram-negative coccus and a bacillus from the rumen of cattle. The bacillus appeared to be the more active cellulose fermenter, but he could not assess the significance of these organisms in the rumen, since the numbers were small. In 1950 Hungate published an extensive account of his investigations with cellulose fermenting bacteria. From the bovine rumen he isolated two Gram-negative bacilli, three strains of a coccus with a white colony and one with a yellow colony. The more active of the bacilli fermented only cellulose and starch or their hydrolysis products; in addition, this organism also formed CO_2, propionic acid, butyric acid and large amounts of succinic acid from cellulose. It was therefore named Bacteroides succinogenes. The less active cellulolytic rod was not identified fully. All three strains of the coccus with white colonies produced CO_2 hydrogen, ethanol, lactic acid, formic acid and acetic acid from cellulose. The yellow coccus after a prolonged incubation (180 days) produced cellobiose in the medium in addition to carbon dioxide, hydrogen, acetic acid, ethanol and lactic acid. It was found that to obtain optimum growth with any of

these cultures, enrichment of the medium with rumen liquid was necessary.

Sjipesteijn (1948) isolated from the rumen of cattle a Gram-positive coccus Ruminococcus flavefaciens and a Gram-negative oval shaped organism Ruminobacter parvum. The latter organism differed from the ruminococcus morphologically, the shape of its cell being oval. Apart from this difference, both organisms were found to be very similar: both digested cellulose, were strict anaerobes, and required certain growth factors which were present in rumen liquid. Succinic acid resulted from the metabolism of cellulose by Ruminobacter parvum while acetic and propionic acids were the products of Ruminococcus flavefaciens. Acetic and propionic acids are found as metabolic products in rumen liquor, while succinic acid is present only in very small amounts. Both organisms grew under conditions similar to those in the rumen. Growth in pure culture in artificial media was poor but an important synergistic effect in mixed culture was noted. It was found that the ruminococcus grew well in artificial culture if grown in conjunction with either Clostridium welchii or Clostridium septicum, while Escherichia coli had a much slighter effect. Sjipesteijn suggested two possible

explanations for this finding of the favourable effect of the synergistic bacteria. It might be due either to the removal by the synergistic organism of harmful metabolic products which otherwise accumulated in the medium or to the addition of some growth factor to the medium by the synergistic strain. Sjipesteijn thought that since the volume of the medium was large in relation to the small quantity of cellulose decomposed it did not seem likely that the synergistic organism removed harmful metabolic products. Since the synergism was specific and the clostridia gave a more pronounced effect than the escherichia it seemed probable that the production of a growth factor was the more satisfactory explanation of the synergism. This finding is of the greatest importance in rumen bacteriology. It may well be that the rumen microflora have, with evolution, become so interdependent that some groups of bacteria cannot live and grow without the presence of others. This finding may also explain many of the difficulties encountered in the isolation of pure bacterial cultures from the rumen.

Quin (1943) observed that an oval shaped micro-organism developed in the rumen of sheep which were fed on a diet rich in soluble sugar.

He considered it to be a yeast which he named Schizosaccharomyces ovis. Studies of McGaughey and Sellars (1948) and Ingram and McGaughey (1948) suggested that since this organism was motile it could not be a yeast and therefore belonged to the genus Selenomonas and was considered by these workers to be identical with Selenomonas ruminantium. Recently Van der Westhuizen, Oxford and Quin (1950) attempted to isolate this organism on yeast medium but were unable to grow it in artificial culture. Although these workers did not succeed in culturing this organism, it has been observed in rumen contents of animals in widely differing geographical localities and is always associated with a diet rich in soluble sugars, so that it is now established as a member of the normal rumen microflora. Van der Wath (1948) isolated an untyped streptococcus from the rumen contents of a sheep fed on a diet containing starch (maize meal). The organism was Gram-positive and stained blue with iodine. It differed from Streptococcus faecalis in several respects and appeared to be more nearly related to Streptococcus bovis. These streptococci were morphologically similar to those digesting starch grains in the rumen. Masson (1951) isolated a strain of Clostridium butyricum from the rumen of a

sheep fed on a diet containing a high percentage of maize starch. This organism was similar, but not identical, to another strain isolated from the pig caecum by Baker, Nasr, Morrice and Bruce (1951). The amylolytic activity of the pig caecum strain was studied by Whelan and Nasr (1951). An investigation of the sheep rumen clostridial amylase and a comparison of its activity with that from pig caecum is described in Section 5.

Johns (1951) studied the fermentation of lactate in the sheep rumen in both England and New Zealand. He isolated one organism, Veillonella gazogenes which was estimated to be present in numbers of 5×10^5 to 77×10^6 per ml. of rumen contents. He found that these organisms, which were Gram-negative diplococci, did not utilise glucose. However lactate was fermented to form propionic acid and when this reaction was carried out in the presence of labelled sodium bicarbonate, it was shown that labelled carbon appeared in the propionic acid. Since these organisms could also decarboxylate succinic acid (at $_pH$ 6.0) to give propionic acid, Johns stated that CO_2 was "fixed" in the lactate to form oxaloacetate which was converted to succinic acid via the 4 carbon acid cycle and subsequently decarboxylated to propionic acid. The rate of the reaction was high

($^{Q}CO_2$ = 270 - 300) and it was thought to be of quantitative importance in the rumen. This paper has been considered in detail because it shows the value which pure culture studies together with biochemical techniques may have in elucidating the complex reactions which can be brought about by the rumen microflora.

Gall and her co-workers in America have published a great deal of information about methods of isolation of bacteria from the rumen of both cattle and sheep. In 1947 Gall, Stark and Looslie published a preliminary survey of the morphological types of bacteria present in the rumen. Gall and Huhtanen (1951) described five rumen bacteria which they had isolated. These organisms were an anaerobic coccus (RO-6TBR), a "very strictly" anaerobic, pleomorphic rod (RO-LI), and a "very strictly" anaerobic, pleomorphic, Gram-positive, branching rod (RO-L2). The last two organisms (RO-L3 and RO-T1) were both anaerobic, Gram-negative, slender rods. The medium used for their isolation was enriched with rumen liquor. The oxido-reduction potential was probably not very low since anaerobic culturing was carried out in tubes sealed with Vaspar. The medium was gassed with carbon dioxide before use but it is doubtful if this would remove

($^{Q}CO_2$ = 270 - 300) and it was thought to be of quantitative importance in the rumen. This paper has been considered in detail because it shows the value which pure culture studies together with biochemical techniques may have in elucidating the complex reactions which can be brought about by the rumen microflora.

Gall and her co-workers in America have published a great deal of information about methods of isolation of bacteria from the rumen of both cattle and sheep. In 1947 Gall, Stark and Looslie published a preliminary survey of the morphological types of bacteria present in the rumen. Gall and Huhtanen (1951) described five rumen bacteria which they had isolated. These organisms were an anaerobic coccus (RO-6TBR), a "very strictly" anaerobic, pleomorphic rod (RO-LI), and a "very strictly" anaerobic, pleomorphic, Gram-positive, branching rod (RO-L2). The last two organisms (RO-L3 and RO-T1) were both anaerobic, Gram-negative, slender rods. The medium used for their isolation was enriched with rumen liquor. The oxido-reduction potential was probably not very low since anaerobic culturing was carried out in tubes sealed with Vaspar. The medium was gassed with carbon dioxide before use but it is doubtful if this would remove

all traces of oxygen and so the culture conditions were perhaps not strictly anaerobic. In this paper Gall and her co-workers put forward a series of criteria for distinguishing a "true" rumen bacterium from an incidental organism. These criteria are: (1) anaerobiosis, (2) presence in numbers of at least 1×10^6 per gram. of fresh rumen contents, (3) at least ten isolations of a similar type bacterium from two or more animals, (4) isolation of similar type bacteria in at least two geographical localities and (5) production by the organism of end-products found in the rumen from substrates found in the rumen. For an organism to be a "true" rumen bacterium, Gall states that it must conform to all these criteria. In 1952 Huhtanen, Rogers and Gall published an improved technique for isolating and purifying rumen organisms in which they used a Brewers anaerobic dish (Brewer 1942). This method had certain advantages over previous methods as it enabled a very rapid differentiation to be made between the strict aerobes, facultative anaerobes and strict anaerobes. The strict aerobes grew only in the region of the air bubble, while cultures of strict anaerobes showed a zone of inhibition round the air bubble but grew well in the region of the dish where the Eh was low. The use of this method

also enabled some of the more delicate anaerobic organisms to be grown from the rumens of roughage fed adult animals. A quantitative estimate of the numbers of bacteria could also be made by this method. Doetsch, Robinson and Shaw (1952) described two organisms which they isolated from the rumen. They found that, because of precipitation, the medium used by Gall was unsatisfactory and they adopted that used by Hungate (1950). Using Hungate's salt solution as a base, two media with different compositions were tried. The more satisfactory one contained 20 ml. Hungate's salt solution, 15 ml. filtered rumen liquor, 0.0001% resazurin and agar to make a solid medium. The medium was sterilised under CO_2 and buffered to pH 7.0 with Na_2CO_3. Sodium thioglycollate, cysteine, glucose and cellobiose were then added as sterile solutions, the first two to keep the Eh low. These workers stated that there was no <u>a priori</u> reason for believing that rumen bacteria were killed off immediately in contact with air. They quoted the work of Pounden and Hibbs (1950), who described how the calf obtained its rumen microflora by infection from the mother, in support of this statement. If rumen organisms are destroyed by exposure to air, the young animal could not develop a normal rumen microflora.

Bryant (1952), using Hungate's technique, isolated a spirochaete from the bovine rumen by using a complex inorganic salt medium with cellulose, cysteine hydrochloride, resazurin and rumen fluid added. The organism was 4 - 14 μ long and had an average diameter of 0.3 - 0.5 μ, with pointed extremities and 4 coils per cell. Morphologically, this organism belonged to the genus Borrelia. The organism is a normal inhabitant of the rumen of cattle and morphologically similar types are present in the ovine rumen. This spirochaete did not digest cellulose but was said to live on the glucose produced by the cellulose decomposing bacteria. Since the spirochaetes were motile they could move through the soft agar and reach the soluble metabolic products which had diffused away from the site of cellulose fermentation and which the non-motile cellulolytic organisms could not reach.

Huhtanen and Gall (1953) in two papers entitled "Rumen Organisms" described the isolation of seventeen different rumen organisms. Two of these were Gram-negative cocci which utilised lactate and appeared to be very similar to Veillonella gazogenes described by Johns (1951). The remaining fifteen strains were described and their properties investigated. The majority of the organisms had

marked fermentative powers, the products of fermentation being lactic, acetic butyric and propionic acids. Vitamin B12 folic acid and pyridoxine were noted as important metabolic products. Taxonomically some of the organisms were stated to show morphological relationships to the genera Propionibacterium, Veillonella and Fusobacterium. No attempt, however, was made to classify these organisms by any of the well known methods.

From this short account of the pure culture studies which have been made on rumen bacteria, it is apparent that we are only beginning to understand the metabolism of the complex microflora of the rumen. Progress has been slow because the classical bacteriological techniques are not suitable and it is only now, with a better understanding of the physiological conditions in the rumen, that new methods can be developed for the study of the rumen bacteria.

THE NUMBERS OF BACTERIA IN THE RUMEN

Since Tappeiner (1882) first appreciated the importance of the role played by the microflora in ruminant digestion, numerous workers have tried to

estimate the number of micro-organisms present in rumen contents. Estimates of the total numbers show great variation. The chief difficulties have not been due so much to the inaccuracies of the methods but rather to the many variable factors on which the numbers depend. It has been established that numbers are subject to both individual and seasonal variation (Moir and Williams, 1951; Moir, 1951).

Moir (1951) found that the density of the ruminal population showed considerable seasonal variation. Using a total counting method he found two distinct levels of population density, the lower associated with dry and the higher associated with green grazing. The peak concentration of 88 million organisms per cu. mm. (88×10^9 per ml.) occurred in the early summer while the lowest number, 34 million per cu. mm. (34×10^9 per ml.), was found in April at the end of the Australian summer. Using a similar technique, Gall, Stark and Looslie (1947) estimated that the total number of organisms in the rumen was $(62 \pm 9.3) \times 10^9$ organisms per ml. Other estimates made by these workers showed that the solid fraction of the rumen contents had a higher total count $(93 \pm 19.5) \times 10^9$ per gram. Hungate (1947) made differential

viable counts on the rumen bacterial population and found a rich coccal population of 8×10^9 organisms per ml. A sarcina was present at a concentration of 3×10^5 per ml. and a cellulolytic rod 5×10^7 per ml. From these figures he calculated that the ratio of cellulolytic to non-cellulolytic organisms was 1 : 1000. A study of animals on other diets showed that the number of cellulose decomposing organisms fluctuated from 2×10^4 to 1×10^9.

Another factor which directly influenced the numbers of bacteria in the rumen was the health of the animal. In bracken poisoning for example, both the total and viable counts were very much reduced (Moir, personal communication).

Although estimates of the numbers of bacteria in the rumen vary enormously it is clear that the density of the rumen bacterial population is greater than that appearing in most other habitats and in ordinary liquid laboratory cultures.

SECTION I

THE ISOLATION AND IDENTIFICATION OF AMYLOLYTIC STREPTOCOCCI FROM THE RUMEN OF SHEEP

Figs. 2 and 3 show the appearance of raw maize and potato starch granules. Individual potato starch grains vary from 10 - 30 μ and maize 10 - 15 μ in diameter. Microscopic examination of the rumen contents of a sheep given a diet containing these starches shows that the grains are surrounded by bacteria, which are mainly cocci of various sizes (Figs. 4 and 5). If the preparation is stained with iodine by the method originally described by Henneberg (1922), a large proportion of the organisms attached to the starch grains can be seen to give a blue reaction. The reaction is due to the endocellular synthesis of a bacterial polysaccharide resembling starch and described by Smith and Baker (1944). These organisms have been termed iodophil by Henneberg (1922) and the term has since been used extensively in rumen bacteriology, particularly by Moir and Masson (1952) in their morphological classification of rumen bacteria.

The carbohydrate requirements of domestic ruminants are largely supplied in the form of cereal

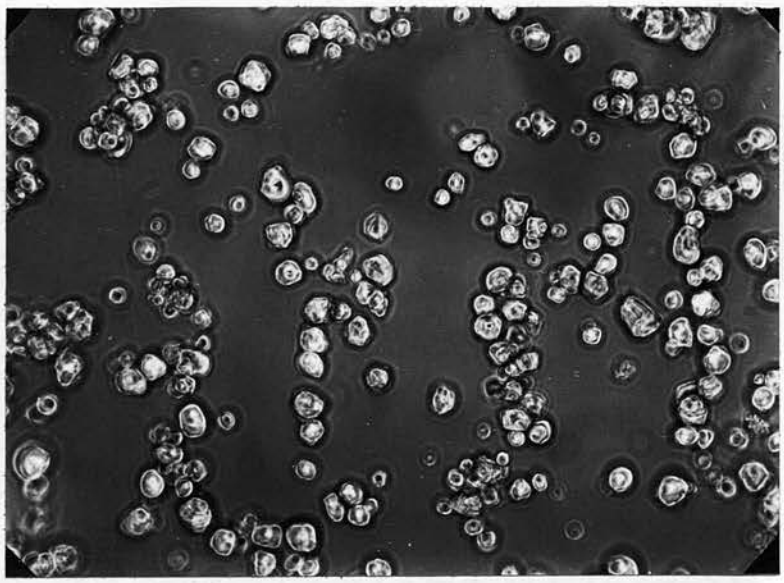

Fig. 2. Raw maize starch granules.
Phase contrast illumination in
water X 270.

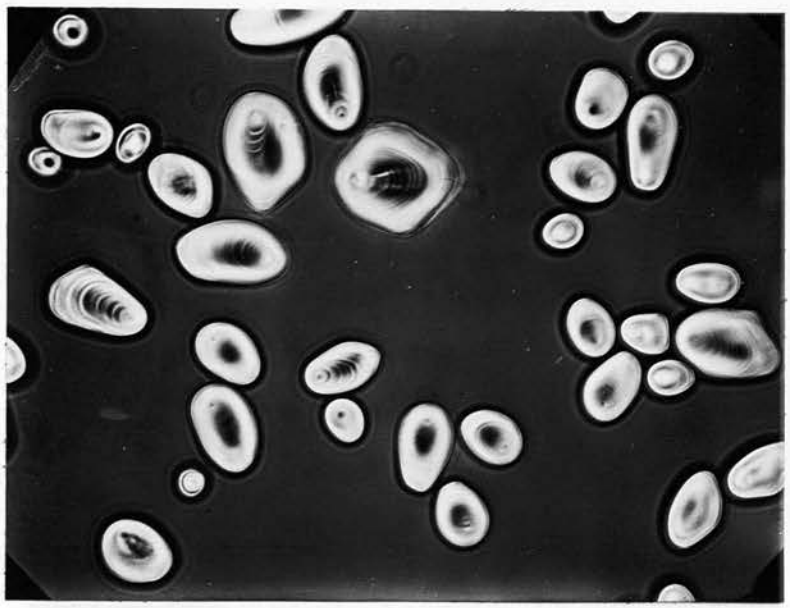

Fig. 3. Raw potato starch granules.
Phase contrast illumination in
water X 270

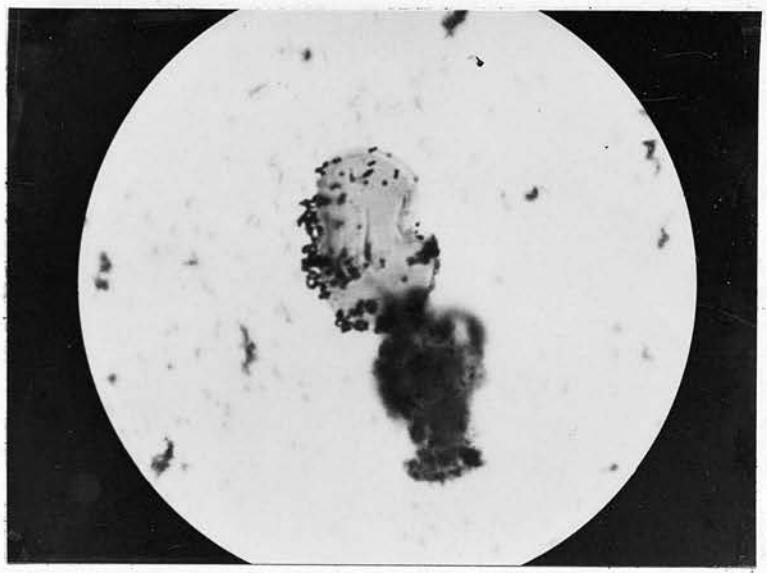

Fig. 4. Maize starch from the rumen of sheep surrounded by digesting bacteria. Methylene blue in wet suspension X 480.

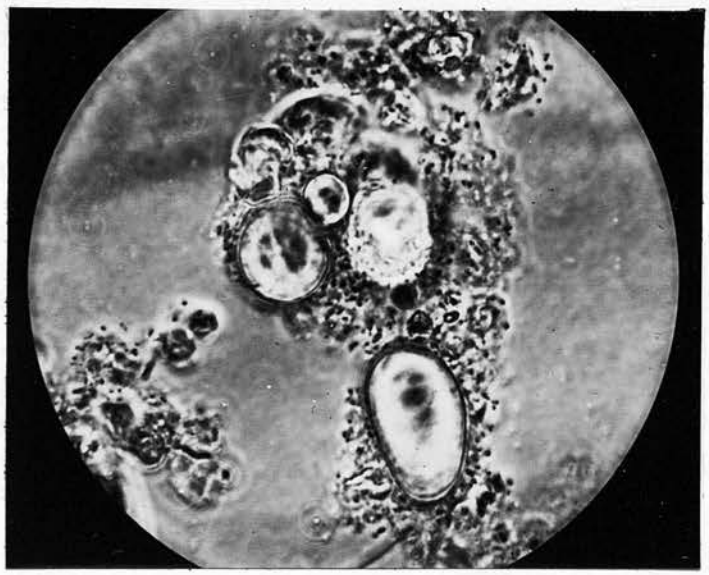

Fig. 5. Potato starch grains from rumen of sheep surrounded by digesting bacteria. Phase contrast illumination in water X 480.

and tuber starches which are usually fed uncooked. Although ruminant saliva contains no amylase and the rumen itself no digestive glands, most of the starch in the diet is digested before it leaves the rumen. Hence, the rôle of the rumen microflora in this initial phase of the carbohydrate metabolism of the ruminant is clearly very important. For this reason a study of the processes of starch digestion and of the organisms involved in these processes was undertaken. In particular, it seemed necessary to attempt the identification of the starch digesting organisms and to settle their relationship to other intestinal bacteria.

The granules of different types of uncooked starch are sufficiently distinct structurally (Figs. 2 and 3) to allow their easy recognition in a mixture of food materials such as is found in the rumen. Thus the fate of the different starches in the alimentary tract can be determined; microscopy may be used to estimate the time taken for digestion to take place and also the morphology of the micro-flora associated with these changes.

The extensive literature dealing with the bacterial decomposition of starch (Thaysen and Galloway, 1930) is largely based on cultural investigations in which soluble starch is used i.e.

starch which has been dissolved by bursting the granules in boiling water. Raw starch granules are far more resistant to bacterial attack than cooked or ground starches (Baker, personal communication) so that results with soluble starch *in vitro* may give an inadequate picture of the changes taking place in uncooked diets.

Stark and Tetrault (1951) stated that the ability to ferment starch was characteristic of a large group of mesophilic bacteria, if the organisms were cultured under suitable conditions. Even slight variation in these optimal conditions might cause cessation of starch fermentation.

The enzymes of the starch disintegrating bacteria act *in situ* upon the surface of the granules. This is possible because the bacteria are firmly attached to the starch grain (Figs. 4 and 5), the mechanism by which this attachment takes place is not yet fully understood and it is difficult to produce this attachment *in vitro*.

The microscopy of starch digestion.

The structural features attending starch digestion constantly include four main changes :-

(1) Indentation and pitting of the surface of the grains. The initial focus of digestion varies: in maize starch it usually begins

at the hilum, while in potato starch grains there is no constant primary site of penetration.

(2) Progressive alteration in the colour reaction of the grain with iodine - the initial blue-black giving place in sequence to mauve and red reactions. In the early stages of starch breakdown the granules may be parti-coloured. As breakdown continues colourless residues are often observed in which the lamellation of the granule is still visible.

(3) The loss of birefringence in polarised light in potato starch. This takes the form of progressive alteration of the maltese cross.

(4) The intensity with which the starch grains stain with congo red is increased.

MATERIAL AND METHODS

Sheep.

Samples for examination were obtained from sheep of the following breeds :- Black face, Grey face (Border Leicester x Black face), Cheviot and Half bred (Border Leicester x Cheviot). The ages of the animals ranged from 2 - 14 years. The sheep were housed in individual pens and each was allowed access

to salt and mineral licks at all times. The animals were fed on a variety of diets, containing various proportions of concentrates and hay, with water ad. lib.

Times of feeding.

The sheep were fed thrice daily with two feeds of concentrates; the hay was given in between. Those animals receiving an all hay diet were fed twice daily at 12 hour intervals, the food and water being removed 2 hours after feeding. This practice prevented any changes occurring in the rumen during the next 10 hours owing to a fresh influx of food and water.

Operations for fitting cannulae.

Rumen cannulae were inserted according to the technique of Phillipson and Innes (1939). The operations in which the ebonite cannulae were inserted were performed under anasthesia by Drs. A.T. Phillipson, D. Duncan and Mr. A. Iggo of the Rowett Research Institute.

Methods of obtaining samples.

The rumen was massaged before sampling in order to obtain a more representative sample of the contents (Tosic, personal communication). Samples were removed from the rumen by inserting a glass tube through the cannula. The size of the glass tube was 0.5 - 1.0 cm. internal diameter, according

to the size of the cannula. Tubes of as high an internal diameter as possible were used since this enabled a more representative sample to be obtained. A large rubber teat attached to one end of the tube provided sufficient suction to fill the tube with rumen contents. Animals without fistulae were sampled by means of a stomach-tube. The sheep's mouth was held open with a mouth-piece and a polythene tube of 1 cm. internal diameter passed through the centre of the mouth-piece. The path taken by tubes passed into the oesophagus was described by Watson and Jarrett (1945), who showed that the tube, unless accompanied by a liquid, passes into the rumen or reticulum. After carefully introducing the tube 27 - 30 inches, negative pressure was applied by means of a large syringe. A trap in the line was employed so that the ruminal sample was withdrawn directly into a large sample bottle. Samples of only about 100 ml. could be obtained by this method (compared with the much larger samples of 1.0 - 1.5 litres which could be obtained from sheep with permanent fistulae). For the sake of standardisation, samples were collected 3 hours after the morning feed when bacterial numbers were reaching their peak (Moir and Williams, 1950).

Treatment of the samples.

To avoid aeration, the conical flask in which the sample was collected was completely filled and CO_2 was bubbled slowly through the sample for 5 minutes before use to ensure that the bicarbonate buffer was restabilised.

Method of isolation of amylolytic streptococci from the rumen of the sheep.

The rumen sample was obtained and treated in the method described above. Approximately 5 grams were diluted with 500 ml. of sterile quarter-strength Ringer's solution. Direct plating out of rumen contents was tried, but since diluted material gave similar results and was much easier to handle, the diluted material was used throughout.

A loopful of the diluted rumen contents was streaked out onto a yeast starch agar medium of the following composition :-

1 per cent. Bacto tryptose

1 per cent. yeast extract (Difco dehydrated)

0.1 % $KH_2 PO_4$

2 % agar.

The final $_pH$ was adjusted to 6.8. After sterilisation of the medium at 15 pounds pressure for 15 minutes, 15 ml. of a sterile 5% potato starch

solution was added to each 100 ml. of medium. This potato starch solution was prepared by continually stirring the starch and water mixture over a boiling water bath until the starch grains burst and the mixture thickened. This solution was sterilised at 5 lbs. pressure for 30 minutes. The final concentration of 0.75 per cent. starch in the medium was sufficient to make the medium opaque.

Plates, which had been inoculated by streaking the diluted rumen contents over the surface of the dried medium, were incubated in an atmosphere of 10 per cent. CO_2 and 90 per cent N_2 and at 39 - 40°C since this temperature is close to that of the sheep rumen. In order to control the atmosphere of incubation, cultures were incubated in McIntosh and Fildes jars. Comparing the colony size of cultures grown in different atmospheres (Figs. 6, 7 and 8) it was found that, if other conditions were kept constant, the colony size of cultures grown in the CO_2 - N_2 mixture was largest while those grown in hydrogen were smallest. The colony size of cultures grown aerobically was intermediate between that in CO_2-N_2 and in hydrogen. This variation in the colony size was only observed in the primary stages of isolation.

After incubation the amylolytic colonies were

Fig. 6. Culture of amylolytic streptococci grown in an atmosphere of 10% CO_2 and 90% N_2.

Fig. 7. Culture of amylolytic streptococci grown in an atmosphere of air.

Fig. 8. Culture of amylolytic streptococci grown in an atmosphere of H_2.

easily recognised by the clear zones which surrounded each colony. That these zones were in fact the result of starch hydrolysis and not some other change was easily demonstrated by flooding the plate with iodine solution; the zones remained clear whereas the unhydrolysed areas of the medium stained blue. The growth of amylolytic streptococci on starch is shown in Fig. 9 in which it should also be noted that the colonies themselves show a starch reaction with iodine. Amylodextrin, an intermediate product in starch hydrolysis, which reacted with iodine to give a red colour, could be seen around the periphery of the zone of starch hydrolysis.

The purification of the cultures.

Single well isolated colonies from the primary culture were selected and sub-cultured on the yeast starch agar. Pure cultures were obtained by selection of single colonies which were alternately sub-cultured in liquid and on solid media.

Strains were isolated by this method and submitted to various biochemical tests recommended for the identification of non-pathogenic streptococci (Breed, Murray and Hitchens, 1948, Sharpe and Shattock, 1952, Shattock, 1949). Details of the sources from which cultures were obtained are shown in Table 1. This table shows that it was found

Fig. 9. Culture of amylolytic streptococci on yeast-starch-agar medium.
Plate flooded with iodine to show zones of starch hydrolysis.

Table 1. Sources of the strains of amylolytic streptococci isolated from sheep rumen.

Strain	Date of isolation	Sheep number	Diet *	Method of sampling **
16A	1.11.50	A	H,C.	S.T.
16B	1.11.50	B	H,C.	S.T.
16C	1.11.50	C	H,C.	S.T.
2SA	11.11.50	A	H,C.	S.T.
BS07	16.11.50	B	H,C.	S.T.
AS06	16.11.50	A	H,C.	S.T.
722B	12. 7.51	722	H,C,G.N.	F.
718A	12. 7.51	718	H,C.	F.
716A	17. 7.51	716	H,C.	F.
1010	15. 8.51	1010	F.M,H.	F.
EA	14. 8.51	722	H,C,G.N.	F.
BA	18. 8.51	1054	F.M,H.	F.
BB	18. 8.51	1054	F.M,H.	F.
DA	15. 8.51	722	H,C.	F.
1004B	14. 8.51	1004	H.	F.
AA	14. 8.51	1054	G.M,H.	F.
EB	14. 8.51	722	H,C,G.N.	F.
1A	15. 1.52	972	H.	F.
1B	15. 1.52	972	H.	F.
2A	15. 1.52	972	H.	F.
2B	15. 1.52	972	H.	F.
2C	15. 1.52	972	H.	F.
3A	15. 1.52	972	H.	F.
3B	15. 1.52	972	H.	F.
2B0	11.11.50	2	H,C.	S.T.
GMA	12. 7.51	1054	G.M,H.	F.
GMB	12. 7.51	1054	G.M,H.	F.
CA	14. 8.51	716	H,C.	F.

* H,C. hay and concentrates
H,C,G.N. hay, concentrates, and ground-nut meal
F.M,H. flaked maize and hay
G.M,H. ground maize and hay
H. hay

** S.T. stomach tube sample
F. fistula sample

possible to isolate these amylolytic streptococci from different sheep on a wide variety of diets, even from animals on an all hay diet which contained only small amounts of soluble carbohydrate.

Fermentation reactions.

These tests were carried out in peptone water containing 1 per cent. of Andrade's indicator and 0.5 per cent. of the test substrate. Since the streptococci under investigation would not grow in peptone water alone 1 drop of sterile sheep serum was added to each tube containing 2.5 ml. of the medium. To ensure sterility, after intermittent sterilisation all tubes were incubated at 37°C for 18 hours. A loopful of an 18-hour nutrient broth culture was used as inoculum and the cultures were incubated for 5 days at 39 - 40°C. After incubation the cultures were examined for acid and gas production and the $_{p}H$ of each culture checked with a B.D.H. capillator and appropriate indicator. A control tube containing no substrate was always included with each set of incubations to ensure that the serum did not contain sufficient fermentable substrate to turn the indicator colour.

Gelatin liquefaction.

Gelatin stabs containing 12 per cent. of gelatin were inoculated from young nutrient agar

cultures and incubated at 39 - 40°C for 1 week. The tubes were cooled for 6 - 8 hours at room temperature before being read.

Heat resistance test.

Eighteen-hour nutrient broth cultures were carefully heated in a water bath at 60°C for 30 minutes. Absence of growth after sub-culture was interpreted as meaning that the heated culture was no longer viable.

Hydrolysis of sodium hippurate.

The organisms were grown in infusion broth containing 1 per cent. sodium hippurate and incubated for 7 days at 39 - 40°C. One ml. of uninoculated medium was tested for the presence of benzoate by the ferric chloride method. The volume of 12 per cent. solution of ferric chloride which just failed to give an insoluble precipitate with 1 ml. of uninoculated medium was determined by titration and this volume was added to 1 ml. of the centrifuged supernatant of the culture. A brown precipitate indicated that the sodium hippurate had been hydrolysed.

Growth at $_{p}$H 9.6. This was carried out by the method described by Shattock and Hirsch (1947).

Rumen liquor medium.

This medium favours the development of

capsules by rumen streptococci, which have been found useful in their serological identification. The medium was prepared by filtering the rumen contents from a sheep on an all hay diet through muslin. The bacteria were removed from the filtrate by centrifuging on a Sharples centrifuge. To 100 ml. of this liquor were added 1 ml. of phosphate buffer, 1 per cent casein hydrolysate, 2 per cent. agar, and 15 ml. of a 5 per cent. starch solution.

The phosphate buffer was made by dissolving 5 grams. KH_2PO_4 and 5 grams K_2HPO_4 in 90 ml. of water. This medium was sterilised at 10 pounds pressure per square inch for 20 minutes.

RESULTS

Amylolytic streptococci were isolated from the rumen contents of sheep on a wide variety of diets including an all-hay diet containing very little starch (see Table 1).

Cultural characteristics of the amylolytic streptococci

Morphology.

Films made from fluid and solid cultures and

stained by Gram's method showed small Gram-positive cocci arranged in pairs and chains (Fig. 10). The length of the chain depended on both the age of the culture and the culture medium. Cultures grown in the serum peptone water containing raffinose grew in very long chains, whereas in contrast to this, culture in cooked meat medium showed few chains and the majority of cells were arranged as diplococci.

The size of individual cells ranged from 1.0 - 1.5 μ in length and 1.0 μ in breadth. All strains were found to grow both aerobically and anaerobically.

Biochemical reactions.

The biochemical reactions given by these organisms are shown in detail in Table 2. The table shows that these bacteria fermented glucose, galactose, sucrose, maltose, lactose, starch dextrin and salicin; they did not ferment adonitol, mannitol, dulcitol, rhamnose, sorbitol, glycerol or inositol. The fermentation of trehalose, raffinose, inulin, aesculin, arabinose and xylose was variable, some strains fermenting these substrates while others did not. The $_{p}H$ of the medium after fermentation was 4.5 except with arabinose and xylose in which it was 6.0 - 6.4.

Gelatin liquefaction.

Gelatin was liquified by five out of 27 strains

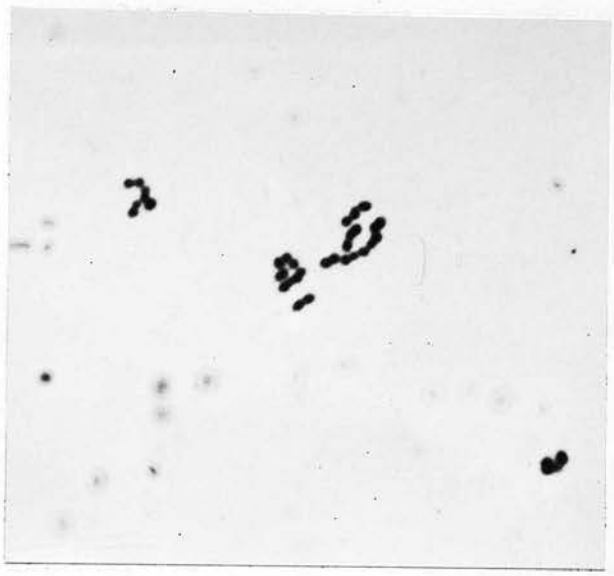

Fig. 10. Morphology of amylolytic streptococci showing small cocci arranged in pairs and short chains.
Broth culture stained by Gram's method X 2700.

Table 2. Fermentation reactions of the strains of amylolytic streptococci isolated from sheep rumen

Strain number	SUBSTRATE																				
	arabinose	xylose	rhamnose	glucose	galactose	sucrose	maltose	lactose	trehalose	raffinose	starch	imulin	dextrin	glycerol	adonitol	mannitol	dulcitol	sorbitol	salicin	inositol	aesculin
2B0	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥ L	⊥	—	—	—	—	—	⊥	—	⊥
GMA	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
2C	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
3A	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
1A	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
2B	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
3B	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
1B	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
2A	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
CA	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	⊥	—	—	—	—	—	⊥	—	⊥
GMB	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
12S01	—	—	—	⊥	⊥	⊥	⊥	⊥	—	—	⊥	—	⊥	—	—	—	—	—	⊥	—	⊥
16A	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
16B	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
16C	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
2SA	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
BS07	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
AS06	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
722B	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥ L	⊥	⊥	⊥ L	⊥	—	—	—	—	—	⊥	—	⊥
718A	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
716A	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
1010	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
EA	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥ L	⊥	—	—	—	—	—	⊥	—	⊥
BA	⊥	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	⊥	—	—	⊥	—	—	⊥	—	⊥
BB	⊥	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	⊥	—	—	⊥	—	—	⊥	—	⊥
DA	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
1004B	—	—	—	⊥	⊥	⊥	⊥	⊥	—	⊥	⊥	⊥	⊥	—	—	—	—	—	⊥	—	—
AA	—	—	—	⊥	⊥	⊥ W	⊥	⊥	⊥	—	⊥	—	⊥	—	—	—	—	—	⊥	—	⊥
EB	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥ L	⊥	—	—	—	—	—	⊥	—	⊥
* S.bovis	—	—	—	⊥	⊥	⊥	⊥	⊥	—	—	⊥	⊥	⊥	—	—	—	—	—	⊥	—	⊥
** S.faecalis	—	—	—	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	—	⊥	—	—	⊥	—	—	⊥	—	⊥

Key to symbols

— ....no fermentation

⊥ ....acid, no gas.

⊥ L ....acid, no gas, late fermenter

⊥ W ....acid, no gas, weak fermenter

*S.bovis ..Streptococcus bovis

**S.faecalis.Streptococcus faecalis

Both of these organisms were obtained from N.C.T.C. and were not isolated from the rumen.

of these organisms (see Table 3).

Heat resistance.

Nine out of twenty-seven strains (33 per cent.) were heat resistant: that is they withstood heating in broth at 60°C. for 30 minutes (Table 3).

Growth at $_{p}$H 9.6.

None of the strains grew at this $_{p}$H. (Table 3).

Hippurate hydrolysis.

Six of these 27 strains hydrolysed sodium hippurate (Table 3).

The results in Section I are discussed at the end of Section 2.

Table 3. Additional properties of the amylolytic streptococci from the sheep rumen

Strain	Gelatin liquefaction	Heat resistance	Growth at $_{p}$H 9.6	Hippurate hydrolysis
AS06	-	-	-	-
16B	-	-	-	-
16C	-	-	-	-
G.M.B.	-	-	-	-
2C	-	-	-	-
2B	-	-	-	-
16A	+	+	-	-
722B	-	+	-	-
718A	+	+	-	+
2SA	-	-	-	-
BS07	-	-	-	-
BB	+	+	-	-
1A	-	-	-	-
EB	-	+	-	-
3A	-	-	-	-
BA	-	-	-	+
716A	-	-	-	-
EA	-	-	-	-
2A	-	-	-	+
AA	+	+	-	-
1B	+	+	-	+
1010Y	-	-	-	-
2B0	-	-	-	-
3B	-	+	-	+
1004B	-	-	-	-
S. bovis	-	-	-	-
S. faecalis	-	+	-	+

SECTION 2

SEROLOGICAL RELATIONSHIPS OF THE SHEEP RUMEN AMYLOLYTIC STREPTOCOCCI

The biochemical reactions of the strains of amylolytic streptococci whose isolation from sheep rumen was described in Section I were very similar. It was decided therefore to investigate their serological properties with the object of elucidating relationships and differences amongst the strains.

METHODS

Preparation of suspensions for the production of immune sera.

Cultures of streptococci which had been carefully purified were grown for eighteen hours at 39 - 40°C in nutrient broth containing 0.2 per cent. glucose. The cells were separated from the culture medium by centrifugation and resuspended in 5 per cent. of the original culture volume in 0.2 per cent. formol-saline. These suspensions were stored at 1°C. for 48 hours. Before use, a

quantity of the formolised cell suspension was added to saline to give a suspension with an opacity equal to Wellcome opacity tube 10 (approx. 3.3×10^9 organisms per ml.). The cells suspended in the saline easily without observable clumps.

Cell suspensions which had been washed three times and killed by heating at 65°C. for 1 hour were also used but the immune sera produced by this method were not so satisfactory as those made from formolised suspensions.

Immune sera.

Antisera were prepared in rabbits by repeated intravenous injection of 1 ml. of the streptococcal suspensions daily for 1 week followed by a week's rest and a further course. The courses were repeated until the agglutinating antibody titre of the serum was above 1 in 640 (Method to be described). When a satisfactory titre was reached, blood was removed from the ear vein and the serum separated with sterile precautions and stored without preservative at 0°C. It was found that, to obtain an unhaemolysed serum, the blood had to be collected from the rabbit quickly. The specimen of blood was then incubated for 20 minutes at 37°C. to facilitate the clot formation and subsequently stored at + 1°C. for 48 hours. This method was found to give the maximum yield of unhaemolysed

serum. Serum prepared in this way kept for at least 6 months.

It was found important to observe the rabbits used for anti-sera preparations at frequent intervals during the period of injections. Adult rabbits were housed in individual cages. Their diet was varied, with standard rabbit pellets and a mixture of bran and bruised corn. Fresh greens were fed daily. White rabbits were found to be the most suitable for production of antisera. They appeared to stand up to the injections more satisfactorily than the other varieties and a suitable antibody titre was obtained more rapidly with this strain. Usually it was necessary to give three courses of injections, but sometimes five were necessary before a suitable serum was obtained. In order to maintain the antibody titre it was necessary to give a boosting dose of four or five injections every four to six weeks, the time varying both with the rabbit and the organism injected.

Preparation of bacterial extracts for precipitin tests.

Grouping extracts were prepared by methods described by Lancefield (1933) and Fuller (1938). The Fuller formamide method was slightly modified in that cells from 50 ml. of culture were used for

preparing the extracts, with proportional increase in all reagents used. The final precipitate was dissolved in 2 ml. of saline. This resulted in a more concentrated extract which gave a more rapid precipitin reaction.

Precipitin tests.

Two drops of antiserum were pipetted into a small agglutination tube and an equal amount of the antigenic extract was layered on top of the serum. The tubes were examined for the presence of a white precipitin ring after 30 minutes at 37°C. In all precipitin tests a control tube was set up in which the antigenic extract was layered on top of normal rabbit serum. This was important in later work, when attempts were made to purify the antigenic components of the bacterial cells and estimate their purity by titration with the precipitin reaction. The protocols for this titration are given in Table 4.

Method of demonstrating capsules. The simplest method for demonstrating capsules was the wet Indian-ink technique first described by Rowland (1914) and later used extensively by Duguid (1948, 1951). However instead of examining the preparation with the ordinary light microscope, phase contrast illumination was used. This enabled the morphology

Table 4. Specimen protocol of the precipitin titration

	Tube	0.85% NaCl	Antigen	Initial total volume in tube	Final total volume in tube	Dilution of antigen
		ml.	ml.	ml.	ml.	
	(1)	0.9	0.1	1.0	0.9	1 : 10
	(2)	0.9	0.1 of (1)	1.0	0.5	1 : 100
	(3)	0.5	0.5 of (2)	1.0	0.5	1 : 200
	(4)	0.5	0.5 of (3)	1.0	0.5	1 : 400
	(5)	0.5	0.5 of (4)	1.0	0.5	1 : 800
Antigen dilutions	(6)	0.5	0.5 of (5)	1.0	0.5	1 : 1600
	(7)	0.5	0.5 of (6)	1.0	0.5	1 : 3200
	(8)	0.5	0.5 of (7)	1.0	0.5	1 : 6400
	(9)	0.5	0.5 of (8)	1.0	0.5	1 : 12800
	(10)	0.5	0.5 of (9)	1.0	0.5	1 : 25600

	Tube	Precipitin serum	Antigen		0.85% NaCl	
			Dilution	Amount		
		ml.		ml.		
	(1)	0.1	1 : 100	0.1	-	
	(2)	0.1	1 : 200	0.1	-	
	(3)	0.1	1 : 400	0.1	-	
	(4)	0.1	1 : 800	0.1	-	
	(5)	0.1	1 : 1600	0.1	-	
Precipitin test	(6)	0.1	1 : 3200	0.1	-	
	(7)	0.1	1 : 6400	0.1	-	
	(8)	0.1	1 : 12800	0.1	-	
	(9)	0.1	1 : 25600	0.1	-	
	(10)	0.1	-	-	0.1 ml.)	Control tubes
	(11)	-	undiluted	0.1 ml.	0.25ml.)	

of cells of the encapsulated organisms to be seen clearly and enabled more accurate measurements to be made (Fig. 11).

The largest capsules (up to 5 μ in transverse diameter) were found when the streptococci were grown on the rumen liquor medium. The colonies also reach maximum size of 2 mm. diameter when grown on this medium. A solid medium, however, was not convenient to use and, since quite large capsules (4.5 to 5 μ) could be demonstrated in young cultures grown in Wright's nutrient broth containing 0.5 % glucose, this medium was normally used for encapsulation studies.

The Neufeld capsule swelling reaction. (Neufeld, 1902).

Cultures were grown in Wright's nutrient broth containing 0.5 % glucose and incubated at 39 - 40°C. for 10 hours. The cells were separated by centrifuging and washed in saline to remove the extra-cellular soluble antigen. The cells were resuspended in saline to give a suspension approximately equivalent to 2.6×10^9 cells per ml. A loopful (5 mm. diameter) of this suspension was placed on a slide and mixed with 2 loopfuls of undiluted antiserum prepared in rabbits as described. A small loopful (2 mm. diameter of a 1 % solution of methylene blue was added. A cover

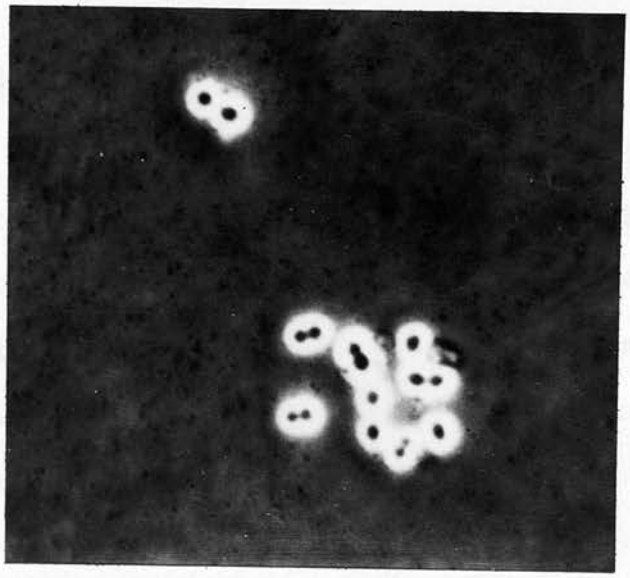

Fig. 11. Encapsulated amylolytic rumen streptococci. The cells can be seen surrounded by large capsules. These latter are represented by clear zones defined by India ink particles. Wet India ink preparation. Phase contrast X 2400.

glass was placed over the mixture and the preparation examined under the microscope. In the presence of homologous immune serum there was an apparent swelling of the capsule without obvious change in the size of the bacterial cells (Fig. 12). It is fairly certain that the immune serum does not cause the capsule to swell but simply alters the consistency or refractive index of an existing capsule so that it becomes optically defined. No such swelling was seen with heterologous or normal sera (Fig. 13). The reaction took place rapidly and the swelling was apparent within a few minutes. The nature of the swelling is not known, but it is specific in that an immune serum produced against one strain will only give a swelling reaction with those strains to which it is antigenically related.

Agglutination titration method.

Preparation of the bacterial suspension.

The cells were grown in Lemco broth containing 1 % peptone (Evans), 1 % Lab lemco, 0.5% NaCl, 0.2% glucose (Analar). The medium was sterilised at 5 lbs. for 30 minutes, the final $_{p}H$ being 6.9 - 7.6. 50 ml. volumes of this medium were inoculated with 1 loopful of a young meat-broth culture and incubated at 39 - 40°C. until an opacity

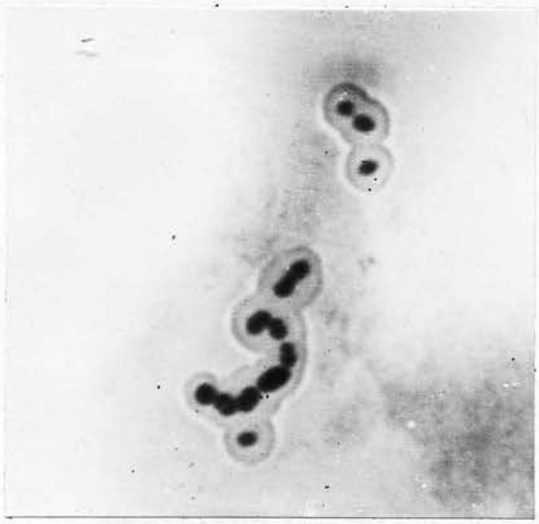

Fig. 12. Streptococci showing a positive Neufeld reaction with homologous antiserum. The "swollen" capsules are seen surrounding the cells and can be demonstrated with the ordinary light microscope.
Wet preparation stained methylene blue
X 2200.

Fig. 13. Streptococci mixed with heterologous serum. No Neufeld reaction. Wet preparation stained Methylene blue X 2200.

approximately equal to Wellcome opacity tube 1 (300×10^6 organisms per ml.) was reached. Usually this took about 9 hours depending on the inoculum. A loopful of the suspension was cultured on blood agar to ensure that it was pure. Then 0.36 ml. of formalin (B.P.40%) (which gave a final concentration of 0.25% of formaldehyde) was added. This suspension was kept at + 1°C. for 24 hours before use. The suspension kept satisfactorily for 4 weeks under these conditions. Until this method of making the suspensions was adopted, considerable difficulty was encountered in obtaining agglutinating titres for the immune sera. As will be discussed in a later section, the metabolic liquid of older cultures contained a considerable amount of soluble antigen which reacted with the sera and prevented agglutination of the cells. Washed cell suspensions were not so satisfactory as the young culture, since it was not possible to get a very even suspension of the centrifuged cells.

Dilution of the sera.

The sera were diluted as in Table 5. The agglutination titrations were carried out in tubes 3 inches x 0.5 inches. The sera were diluted, the bacterial suspensions added and the two thoroughly mixed and incubated overnight at 50°C. in a water

Table 5. Specimen protocol of the streptococcal agglutination titration

Tube	Saline mls.	Serum	Dilution of serum	Bacterial suspension mls.	Final dilution of serum	Final volume in each tube
0	1.4	0.1 ml. undiluted	1 in 15	-	-	
1	-	0.4 ml. tube 0	1 in 15	0.4	1 in 30	0.8 ml.
2	0.4	0.4 ml. tube 0	1 in 30	0.4	1 in 60	0.8 ml.
3	0.4	0.4 ml. tube 2	1 in 60	0.4	1 in 120	0.8 ml.
4	0.4	0.4 ml. tube 3	1 in 120	0.4	1 in 240	0.8 ml.
5	0.4	0.4 ml. tube 4	1 in 240	0.4	1 in 480	0.8 ml.
6	0.4	0.4 ml. tube 5	1 in 480	0.4	1 in 960	0.8 ml.
7	0.4	0.4 ml. tube 6	1 in 960	0.4	1 in 1920	0.8 ml.
8	0.4	0.4 ml. tube 7 Discard 0.4 ml. tube 8	1 in 1920	0.4	1 in 3840	0.8 ml.
control no serum	0.4	-	-	0.4	-	0.8 ml.

bath. The tubes were allowed to cool for 2 hours at room temperature before reading. The results were divided into three groups :-

(1) Complete agglutination in which the cells had settled to the bottom of the tube in large floccules leaving a clear supernatant.

(2) Agglutinated particles or clumps of bacteria which were clearly visible on shaking the tube and examining with a hand lens against a dark surface. The particles remained suspended and did not settle out of the fluid, this result was read as positive.

(3) No agglutinated particles visible after incubation - a negative result.

Viable counts of amylolytic streptococci in sheep rumen contents.

These were done by a roll-tube counting method devised by Dr. A. E. Oxford (Rowett Research Institute). Two sheep were used, one on a diet of 100 g. starch per day together with concentrates and hay, the other on hay only. Samples were taken 3 hr. after feeding. One g. of rumen contents was serially diluted with 9.0 ml. of sterile, neutral 0.25% cysteine in glass-distilled water. Dilutions of up to 10^{-5} were prepared. 0.02 ml. of each dilution was dropped into each roll-tube containing

1.5 ml. yeast-starch-agar. The medium was maintained at 46.5°C. in a water-bath before inoculation. After inoculation the tubes were rolled horizontally under running cold water and were then incubated anaerobically for two days at 39°C. The number of amylolytic colonies were then counted.

RESULTS

Results of the biochemical reactions.

Results of the biochemical reactions show that these rumen streptococci are most nearly related to Streptococcus bovis. Some strains however differ from this in being heat resistant and in this respect appear to resemble Streptococcus faecalis so that a direct comparison can be made between Streptococcus faecalis, Streptococcus bovis and the rumen streptococci. The reactions of these two N.C.T.C. cultures are included in Tables 2 and 3.

Biochemically the rumen strains may be divided into three, those which resemble Streptococcus faecalis or Streptococcus bovis and those which differ from both of these organisms. Streptococcus bovis did not ferment trehalose but it is interesting to note that the majority of rumen streptococci fermented this substrate and so resembled Streptococcus faecalis. However of the 21 strains which fermented trehalose only 8 were heat resistant

and so differed from Streptococcus faecalis. Unlike Streptococcus faecalis the majority of strains fermented inulin and so resembled Streptococcus bovis. Therefore it appears that although the majority of the strains isolated resemble either or both Streptococcus bovis or faecalis they differ from them in minor characteristics. These differences were confirmed by serological reactions.

Viable counts of amylolytic streptococci.

The rumen contents of a sheep receiving a supplement of starch contained approximately two thousand times more starch-splitting streptococci per Gram than were present in the rumen contents of a hay-fed sheep. (4×10^7 viable cocci/g. and 2×10^4 viable cocci/g. respectively).

Capsule sizes.

Table 6 shows that definite capsules were found in the majority of strains isolated. The capsules varied in size but this variation might be due, in part, to variations in the size of the initial inoculum of the culture. A large inoculum led to a more rapid utilisation of nutrient materials with consequent decrease in capsule size.

The Lancefield and Fuller grouping tests.

The results with streptococcal group extracts depended partly on whether the extracts of rumen

Table 6. The comparative sizes of capsules of streptococci grown in glucose nutrient broth.

Strain	Transverse measurement of capsule ø u
2SA	1.75
3A	1.75
BS07	2.1
2C	1.4
1A	1.75
2B	1.4
3B	1.4
16C	2.1
1B	0.7 ⁂
1010Y	1.75
1004B	1.4
16B	1.4
2A	1.0 ⁂
GMA	1.75
GMB	1.75
EA	1.0 ⁂
2B0	1.4
BA	1.0 ⁂
716A	1.0 ⁂
AA	1.4
DA	1.4
16A	1.75
AS06	1.4
722B	1.4
S. bovis	1.4

ø Each figure is the mean of 10 readings

⁂ Encapsulation doubtful

streptococci were made by the acid-extraction method of Lancefield (1933) or the formamide-extraction method of Fuller (1938).

Both types of extract gave a positive precipitin reaction with homologous antiserum. Acid extracts, however, showed no positive reaction with a commercial group-D serum whereas formamide extracts gave a positive precipitin test. Neither type of extract gave any reaction with streptococcal groups - A - B - C - E - F - G - H - K or N sera.

Both the acid and the formamide extracts of Strept. bovis (N.C.T.C. 8177) and Strept. faecalis gave positive reactions with the group-D serum.

Precipitin tests carried out with untreated cell-free supernatants from 18 hour cultures of various strains of rumen streptococci showed strong precipitin reactions with the homologous antisera in dilutions of up to 1 in 10 of the antigen. This antigen, which appeared to be completely soluble, showed the same precipitin reactions as formamide extracts of whole cells. Work on the purification and chemical nature of this antigen, which is probably a capsular polysaccharide, is described in Section 4.

Agglutination titration results.

This method was used for two purposes firstly to determine the agglutinin titres of the immune sera

during production in the rabbit and secondly to determine the relationship of one organism to another.

Some typical results of agglutinin titres of rabbits' sera are shown in Table 7.

In Table 8 are shown the cross agglutinations obtained using different organisms and their sera. It will be seen that some organisms had similar antigenic components and their sera showed cross-agglutinations. For example, organisms BS07 and 16C showed antigenic similarity, while Strept. bovis antiserum and culture were distinct from all the other strains.

Agglutination absorption titrations were carried out on all streptococci showing antigenic relationships in the agglutination titrations. These agglutination absorption titrations were carried out by the method of Colebrook (1935). Streptococci showing antigen relationships were 2B and 2C, 3A and 3B, BS07 and 16C. The minimum absorbing dose for each organism was determined and is shown in Table 9. The sera for the absorptions were standardised by dilution to 60 times the known titre: that is the concentrated sera were diluted 1 in 16 if the agglutination titre was 1 in 960 or 1 in 8 if the agglutination titre was 1 in 480.

Table 7. Agglutinin titres of rabbit antisera for amylolytic streptococci

Antigen	Dilution of serum									
	1/30	1/60	1/120	1/240	1/480	1/960	1/1920	1/3840	control	Titre
2C	+	+	+	+	+	+	+	-	-	1/1920
2SA	+	+	+	+	+	+	+	+	-	1/3840
16C	+	+	+	+	+	+	-	-	-	1/960
3A	+	+	+	+	+	+	+	+	-	1/3840
BS07	+	+	+	+	+	-	-	-	-	1/480
3B	+	+	+	+	+	+	+	-	-	1/1920
2B	+	+	+	-	-	-	-	-	-	1/120
1A	+	+	+	+	+	+	-	-	-	1/960
1B	+	+	+	+	+	+	-	-	-	1/960
S. bovis	+	+	+	+	+	-	-	-	-	1/480

(+ . . . . . agglutination)

Table 8. Cross agglutination reactions in amylolytic streptococci

Culture	Antisera										
	BS07	2SA	2C	3A	3B	1A	16C	1B	2B	16A	S.bovis
BS07	+						+	+			
2SA		+	+								
2C	+		+					+	+		
3A				+	+	+					
3B				+	+						
1A						+		+			
16C	+						+	+			
1B								+			
2B	+		+		+			+	+		
16A		+								+	
S. bovis											+

(Only positive reactions are recorded. All blanks should be read as negative)

Table 9. Minimum absorbing doses of streptococcal suspensions for agglutination absorption titrations

Sera	Density of suspensions				Minimum absorbing dose
	20,000/ml.	10,000/ml.	5,000/ml.	2,500/ml.	
2C	-	+	+	+	20,000/ml.
2B	-	+	+	+	20,000/ml.
3A	-	-	-	-	2,500/ml.
3B	-	-	+	+	10,000/ml.
BS07	-	-	-	+	5,000/ml.
16C	-	-	+	+	10,000/ml.

The results of the agglutination absorption titrations are given in Table 10. It will be seen that complete absorption was obtained with all suspensions after 2 hours at 37°C. with one exception that of serum 3A and antigenic suspension 3B. In this case absorption was repeated three times and resulted in some reduction of the agglutinin titre, but not complete absorption. The percentage absorption was estimated by the method of Colebrook in which a 5 tube reduction of agglutination was calculated as being a 96.8% absorption, while a 3 tube reduction was equivalent to an 87.5% absorption.

The fact that 3A antigen absorbed 3B serum but 3A serum did not completely absorb 3B antigen was probably due to some slight difference in the antigenic structure of the two cultures not demonstrated by other methods.

The Neufeld capsule swelling reaction.

The specificity of the Neufeld reaction is well shown by the results in Table 11. The results are very similar to those of the cross-agglutination reactions which were shown in Table 8. This great similarity makes it likely that both reactions depend on the same antigenic component.

In the Neufeld reaction, as in the cross-

Table 10. Agglutination absorption titrations of antigenically related streptococci

Serum absorbed	Absorbing suspension	Titre of serum before absorption	Reduction of titre after absorption
3A	3A (2,500/ml.)	1 : 480	96.8 % 5 tube reduction of titre
3B	3B (10,000/ml.)	1 : 480	96.8 % 5 " " " "
3A	3B (10,000/ml.)	1 : 480	87.5 % 3 " " " "
3B	3A (2,500/ml.)	1 : 480	96.8 % 5 " " " "
2B	2B (20,000/ml.)	1 : 960	96.8 % 5 " " " "
2C	2C (20,000/ml.)	1 : 960	96.8 % 5 " " " "
2B	2C (20,000/ml.)	1 : 960	96.8 % 5 " " " "
2C	2B (20,000/ml.)	1 : 960	96.8 % 5 " " " "
BS07	BS07 (5,000/ml.)	1 : 480	96.8 % 5 " " " "
16C	16C (10,000/ml.)	1 : 960	96.8 % 5 " " " "
BS07	16C (10,000/ml.)	1 : 960	96.8 % 5 " " " "
16C	BS07 (5,000/ml.)	1 : 480	96.8 % 5 " " " "

Table 11. The Neufeld reactions of amylolytic streptococci

Culture	Antisera										
	BS07	2SA	2C	3A	3B	1A	16C	1B	2B	16A	S.bovis
BS07	+						+	+			
2SA		+	+						+		
2C	+		+					+	+		
3A				+	+						
3B				+	+						
1A						+					
16C	+						+	+	+		
1B	+							+			
2B	+		+					+	+		
16A		+								+	
S. bovis											+

(Only positive reactions are recorded. All blanks should be read as negative)

agglutination reactions, Strept. bovis gave no positive tests with antisera to any of the strains of rumen streptococci and, conversely, its antiserum gave no Neufeld reactions with these organisms.

As was expected commercial Group-D serum did not give a positive Neufeld reaction with any strain of rumen streptococcus.

Although evidence suggests that the Neufeld reaction is as highly specific as other serological reactions, little is known about the theoretical background of the reaction. On examination with ordinary illumination of fluid cultures stained with methylene blue and mixed with normal rabbit serum, no distinct capsular zones can be seen surrounding the cells. Capsules can however be demonstrated around these organisms by the special technique already described using wet Indian ink and phase-contrast illumination.

Application of the Neufeld reaction to the identification of rumen streptococci in situ.

Since finding that streptococci isolated from the rumen were encapsulated and gave a positive Neufeld reaction when mixed with homologous antisera (Figs. 12, 13), it seemed possible that, provided the rumen contents themselves did not inhibit the

reaction, the method might be of great practical value for the identification of encapsulated rumen bacteria *in situ*. Fig. 14 shows a preparation in which a quantity of a pure culture of a streptococcus was mixed with rumen contents. After mixing, homologous antiserum was added, and the swollen capsules of the streptococci could be clearly seen. The morphology of the cells showing a positive Neufeld reaction was distinct from all the other organisms in the preparation. This enabled them to be distinguished from other rumen bacteria including streptococci not showing this reaction but present in the same preparation.

By mixing untreated rumen contents and immune serum together streptococci with swollen capsules could be recognised (Fig. 15). The demonstration of swollen capsules in rumen contents was, however, somewhat unsatisfactory until special methods were adopted for preparing bacterial fractions of rumen contents in which capsules were still present on the bacteria. This method is fully discussed in Section 4.

In an attempt to facilitate the demonstration of a Neufeld reaction in rumen contents, pooled sera were used. It was found, however, that if more than two sera were pooled, the pooled serum no

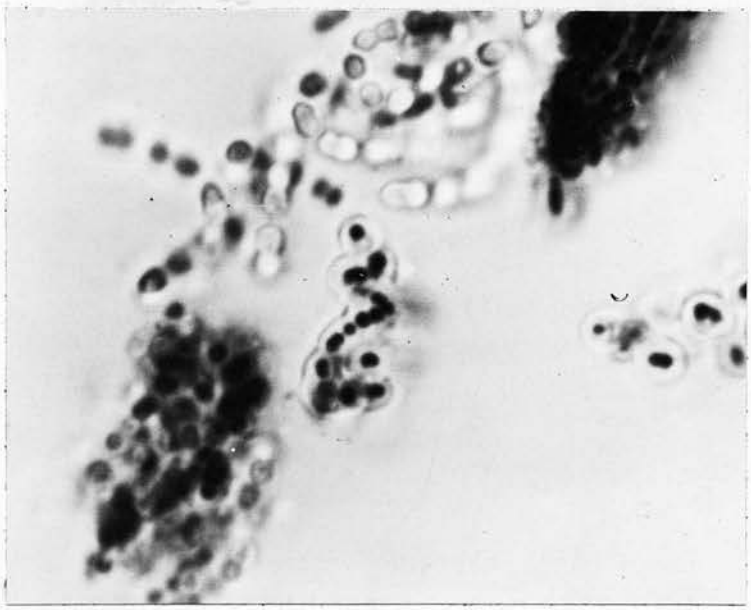

Fig. 14. A positive Neufeld reaction given by a pure culture of amylolytic streptococcus in the presence of rumen contents. Phase contrast X 2300.

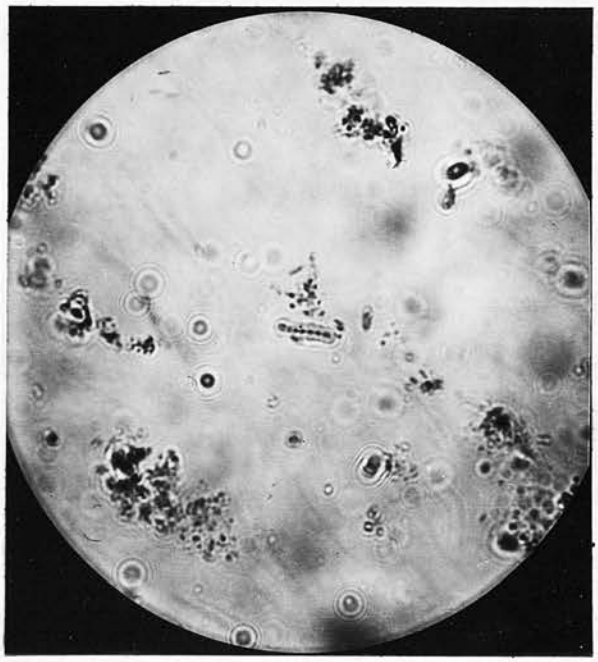

Fig. 15. A positive Neufeld reaction given by amylolytic streptococci <u>in situ</u> in rumen contents.
Wet preparation methylene blue, X 1650.

longer gave a positive Neufeld reaction with all of the pure culture strains. Since the organisms digesting starch in the rumen occur in very dense clusters around the granules, it was found difficult to demonstrate a capsular reaction in these organisms and the most obvious reactions were seen in chains of streptococci floating free in the rumen liquid. It is only possible by this method to examine a minute sample of the rumen contents at any one time and, although Neufeld positive organisms are not very numerous in a microscopic preparation, the serological strain may be quite numerous per gram of rumen contents. Another point which must be borne in mind is the specificity of the reaction - only homologous streptococci will show a positive reaction.

The capsules demonstrated by the Neufeld reaction in rumen contents were usually much smaller than those in pure culture. This was possibly due to the constant churning and grinding to which the organisms were subjected in the rumen.

Since the type specific immune sera have been shown to cause swelling both with pure cultures and with morphologically similar organisms in rumen contents, it may be postulated that there is in the rumen a species of streptococcus serologically

similar to the organisms grown in pure culture and described in this paper. The isolated strains of amylolytic streptococci, therefore, may be accepted as true rumen organisms.

DISCUSSION

The amylolytic streptococci isolated from the rumen appear to be most nearly related to Strept. bovis described by Shattock (1949). Gall and Huhtanen (1951) isolated a streptococcus designated RO-6TBR from the rumen of cattle and sheep. This organism had similar fermentation reactions to those reported here, but it was described by Gall and Huhtanen as a strict anaerobe. The rumen streptococci described in this paper, however, were facultative anaerobes and therefore might not be identical with that described by Gall and Huhtanen. Gall and Huhtanen (1951) stated that all true rumen bacteria must be anaerobic. The ability of the streptococci reported here to grow aerobically should not prevent their being called "true" rumen bacteria since they were capable of fermenting carbohydrates with equal rapidity either aerobically or anaerobically. Viable counts showed that these amylolytic streptococci were present in

the 10^{-6} dilution of rumen contents. It was also shown that similar amylolytic streptococci were not present in the diet and were not introduced into the rumen with the food. These organisms may be assumed, therefore, to be a functional part of the rumen microflora. Since it was possible to isolate them from rumen contents of sheep fed on hay, which was very low in starch, they probably carry out metabolic processes other than starch digestion which may also be of importance in the digestion of food in the rumen.

The starch grains in the rumen could be seen to be surrounded by a bacterial flora which was composed mainly of coccal forms, but pleomorphism was so common among rumen bacteria that it would be unwise to assume, on morphological grounds alone, that the organisms seen in the rumen contents and those isolated in pure culture were identical.

Like many other intestinal streptococci the rumen streptococci appear to belong to Lancefield's group-D, although the Lancefield acid-extraction method was not satisfactory for the serological grouping of these organisms. Concentration and precipitation of the antigen with alcohol (Shattock, 1949) did not increase the reactivity enough to give a positive precipitin reaction with

group-D serum. By using the same serum and group extracts prepared by the Fuller's (1938) formamide method, positive precipitin reactions were obtained. The formamide method therefore appears to be more suitable than the acid method for preparing grouping extracts of these organisms.

Using type-specific sera and the Neufeld reaction it was possible to distinguish different strains of rumen streptococci which showed similar biochemical reactions. Strept. bovis did not give a positive swelling reaction with the antisera of the sheep rumen streptococci and therefore the rumen streptococci appear to be antigenically distinct from this organism.

The morphological change which occurred in the Neufeld reaction allowed the identification of organisms in situ in rumen contents. It had a great advantage as a typing technique over other methods employing specific agglutination since bacteria which were agglutinated but not swollen could not be distinguished from clumps of organisms which normally occur in rumen contents. Since it has been found possible to type other encapsulated bacteria by this method (Carter, 1952; MacPherson, 1948) it is hoped that this technique may have extended application in the identification of

rumen bacteria *in situ*.

SUMMARY

1. Twenty-five strains of amylolytic streptococci have been isolated from the rumen contents of sheep on a variety of diets.
2. In fermentation reactions they have been shown to resemble *Strept. bovis* and a coccus designated RO-6TBR isolated from the rumen by Gall and Huhtanen.
3. By preparation of Fuller's formamide extracts the organisms have been shown to belong to Lancefield's group-D.
4. The cell-free supernatant from young cultures without chemical treatment showed precipitin reactions with the homologous antiserum.
5. The Neufeld reaction has been used to differentiate between the isolated strains and to identify rumen streptococci *in situ* in rumen contents.

SECTION 3

ENCAPSULATION IN RUMEN BACTERIAL FRACTIONS

Methods of fractionating the rumen contents of sheep previously described in the literature (see, for example Smith and Baker, 1944; Johnson et. al., 1944; Heald, 1951) involve centrifugation of uncooled, strained, rumen liquids at varying speeds on centrifuges running at room temperature. These procedures were found to be unsatisfactory for two reasons. First, during the time between collection and centrifugation of the liquids, the composition of the sample might alter as a result of bacterial fermentation and autolysis; and secondly, during the centrifugation, especially at high speeds, the liquids warm up to as much as 40°C., thus setting up convection currents in the centrifugate and causing fermentation with the liberation of gases which break up the sediments. It was found that these difficulties were overcome by the following procedure, which allowed of fractionation on a small scale.

METHODS

Rumen contents were collected through a permanent rumen fistula (Phillipson and Innes, 1939) by means of a wide-bore glass tube fitted with a rubber suction bulb, and immediately pipetted into a conical flask cooled in a freezing mixture of ice and salt, which reduced the temperature of the rumen contents to c. + 1°C. in 10 - 15 min. Further operations were then carried out in a cold room at + 1°C. The rumen contents were strained through a 60-gauge copper gauze to remove plant particles. (It is well to note that, if the fractions are required for cultural studies, the copper gauze should be replaced by bolting silk, as small traces of copper have a bactericidal effect.). The resulting liquid was centrifuged for 20 min. at 22,500 g in a 'Superspeed' head on a refrigerated centrifuge (M. S. E., London), conditions being adjusted so that the rumen liquid remained at + 1° to + 3° during centrifugation. If the temperature was further lowered, ice began to separate from the liquid.

Two sheep were used in the experiments. One was fed mixed concentrates (250 gm.) and potato starch (100 gm.), twice daily, with one feed of hay

(250 gm.) in between, and the other was fed on hay (ad. lib.). The rumen contents were sampled three hours after the morning feed.

RESULTS

The samples from the starch-fed sheep were thick and formed four distinct fractions on centrifuging. These are described in Table 12.

Fractions 1 and 2 from the rumen liquids of the hay-fed sheep were similar to those from the starch-fed animal, but fractions 3 and 4 formed a single layer. The sediments were easily separated by repeated washing with a small volume of phosphate buffer (1.0 M; $_{p}$H 7.0). A photomicrograph of a typical bacterial fraction (2) from the rumen liquids of a starch-fed sheep is shown in Fig. 16.

Examination of fraction 2 by Rowland's Indian ink technique (Rowlands, 1914) showed that large numbers of the bacteria were heavily encapsulated, a fact which has not been noted in fractions prepared by the usual methods (Fig. 17). The largest capsules, up to 9 μ in transverse diameter, were found on the bacteria from the hay-fed sheep. In the fraction from the starch-fed sheep large clumps of agglutinated bacteria surrounded by

Table 12. Fractionation of rumen contents of sheep by high-speed cold centrifugation

Fraction No.	Appearance	Constituents
1	Brown opalescent liquid	Almost bacteria-free; few Gram-positive cocci and Gram-negative bacilli
2	White semi-solid layer	Almost entirely Gram-negative bacilli, cocci and vibrios; few Gram-positive cocci and bacilli
3	Fine green compact sediment	Protozoa, selenomonas and bacteria, as in fraction 2, plant particles and starch granules
4	Coarse material	Mostly plant particles, with some bacteria and protozoa

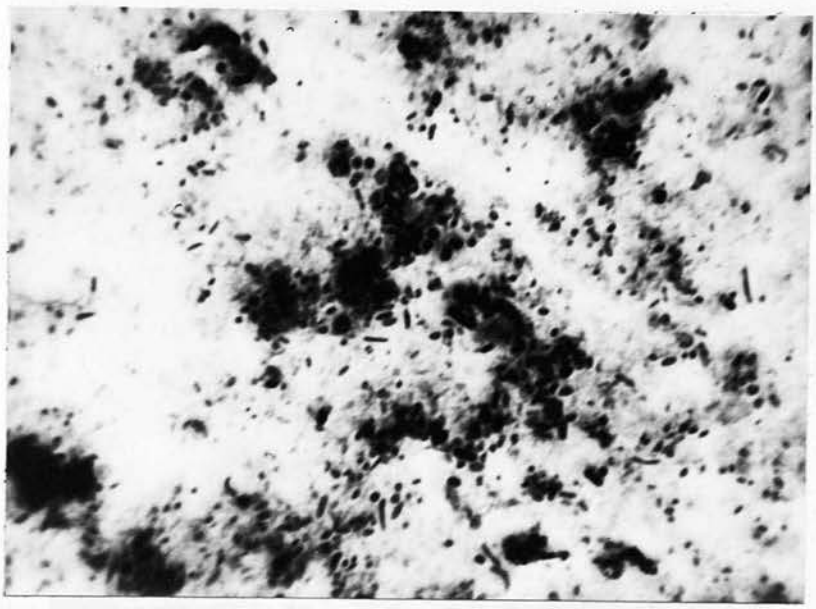

Fig. 16. Bacterial fraction 2 from a sheep on a starch-containing diet.
Stained by Gram's method. X 1000.

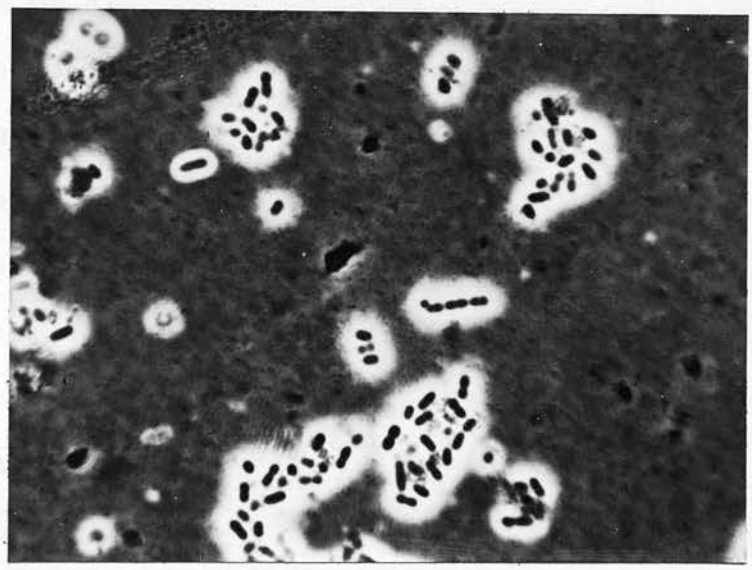

Fig. 17. Bacterial fraction 2 from a sheep on a starch-containing diet. Wet Indian ink preparation, more dilute than Fig. 16 showing extensive encapsulation. Phase contrast. X 2200.

capsular material were seen.

DISCUSSION

Moir and Masson (1952) have recently described a survey of whole rumen contents, either fresh or incubated *in vitro* for one hour with glucose, in which they noted some encapsulated organisms. The present work suggests that encapsulated organisms constitute a large part of the bacterial fractions. This capsular material appeared, from preliminary experiments on the strain of rumen streptococcus isolated in pure culture, to contain polysaccharide which, like the majority of the bacterial capsular materials so far investigated, did not stain with iodine. However, whether this capsular material can be utilized by the host animal at a later stage in the digestion cycle, as can starch-type intracellular polysaccharides (Baker, 1942), or can be converted by other bacteria into products of value to the host cannot yet be stated.

SECTION 4

THE SEROLOGICALLY ACTIVE CAPSULAR POLYSACCHARIDE FROM THE AMYLOLYTIC STREPTOCOCCUS BS07

It was shown in the preceding section that encapsulation is common in rumen bacteria *in vivo*. The amylolytic streptococci, whose isolation was described in Section 1, were found to be encapsulated. In the serological identification of these organisms (Section 2), the Lancefield (1933) and Fuller (1938) methods of extracting antigens were found to give materials which were not entirely comparable, although either method is commonly used for grouping pathogenic streptococci. It was decided, in collaboration with Dr. P. N. Hobson, to investigate the chemical nature of these antigens.

It was also shown in Section 2 that the capsules of these bacteria reacted with specific antisera, giving both positive agglutination and Neufeld reactions. The cell-free supernatant of growing cultures contained material which gave a positive precipitin reaction with the homologous antiserum.

The majority of bacterial capsules, with the

exception of those of some bacilli, are composed mainly of polysaccharide. In a number of cases it has been shown that polysaccharide confers specificity for the organism (Kendall, Heidelberger and Dawson, 1937; Heidelberger, Kendall and Sharp, 1936; Morgan, 1937).

It was decided, therefore, to examine the polysaccharides in the bacterial capsules in the Lancefield and Fuller extracts, and in the medium in which the bacteria had grown ("metabolic liquid").

EXPERIMENTAL

METHODS AND MATERIALS

Analytical methods.

Before analysis the preparations were dried to comstant weight in vacuo at 60° over P_2O_5. In all determinations standard solutions were included along with the unknowns to calibrate the reagents.

(a) Reducing sugars. Reducing sugars were determined using Somogyi's (1952) micro copper reagent in conjunction with colorimetric reagent (Nelson, 1944) calibrated against glucose.

(b) Rhamnose. Rhamnose was measured by a micro-spectrophotometric method (Dische and Shattles, 1949) standardised against rhamnose monohydrate.

(c) Glucosamine. This was determined with the reagents of Elson and Morgan (1933) calibrated with a glucosamine hydrochloride, and using a photoelectric colorimeter.

(d) Nitrogen. Total nitrogen was determined by a micro-Kjeldahl method.

(e) Uronic acids. Uronic acids were determined by the modification of the method of Hanson, Mills and Williams (1944). The reagents were calibrated against a mixture of galacturonic acid, galactose and rhamnose in the approximate concentrations of the polysaccharide to obviate any effects of the sugars on the reagents.

(f) Ash content. The ash content was determined by incinerating a little of the material, in a platinum boat, to constant weight in a micro-muffle.

(g) Phosphorus content. Phosphorus was determined by the method of Allen (1940) after total combustion of the material with perchloric acid.

(h) Acid hydrolysis. This was carried out by

heating the material (concentration approx. 0.5 mg./ml.) with 1.5N - H_2SO_4 in a boiling water bath in a lightly stoppered flask. For reducing sugar determinations the hydrolysate was neutralised with 5N-NaOH and diluted to a standard volume.

(i) Chromatograms. The hydrolysates were neutralised with barium carbonate, filtered, shaken alternately with small portions of anion- and cation-exchange resins (Amberlite IR100/8 and IR4B; Rohm and Hass Co., Philadelphia, U.S.A.) and evaporated to dryness. The residue was dissolved in an appropriate small volume of water and a suitable amount applied to the starting line of a strip of Whatman No. 1 paper along with reference sugars. The chromatograms were developed with the top layer of a benzene, butanol, pyridine, water mixture (in the proportions by volume 10 : 50 : 30 : 30) for 24 hr. at room temperature. After drying the sugars were coloured by spraying with a benzidine reagent (Bacon and Edelman, 1951). Other reagents were used to try to demonstrate the presence of ketoses or to colour hexosamines.

(j) Demonstration of amino acids in HCl hydrolysates. Chromatograms were developed with a phenol, collidine solvent and coloured with ninhydrin. The presence of amino acids indicated that the high nitrogen content of some of the fractions was, at least partly, due to the presence of protein. No attempt was made to identify the amino acids present.

(k) Attempted identification of the uronic acid. Various spectrophotometric methods were tried without success to identify the uronic acid present in the whole polysaccharides and hydrolysates but did not prove satisfactory.

Chromatograms of partially purified acid hydrolysates of the polysaccharide were run in various solvent mixtures, along with glucuronic, galacturonic and mannuronic acid controls. All the chromatograms showed a spot close to that given by the galacturonic acid control and no spots corresponding to the faster moving glucuronic or mannuronic acids were found. The chromatograms were, however, difficult to interpret since the spots were ill-defined and streaky due to the presence of degradation products in the hydrolysate. The identification of the uronic acid present with galacturonic acid is, therefore, only provisional.

Negative staining of the capsules by the wet Indian ink method.

The films were prepared as described by Mackie and McCartney (1950) and examined by phase contrast illumination. Before making the films the $_{p}H$ of the sample was adjusted to 7 to prevent agglutination of the ink particles. High salt concentrations were also found to agglutinate the ink.

Preparation of sera.

The sera were obtained from rabbits after a course of intravenous injections of formolised suspensions of the organisms over a period of some weeks, as described in Section 2. Group-D serum was obtained from Burroughs Wellcome & Co., London. The homologous immune sera were standardised before use by agglutination of a standard cell suspension.

Precipitin tests.

Serial dilutions of the appropriate antigenic material in neutral solution were prepared and layered on top of the immune sera in "Widal" agglutination tubes and after incubation for 30 min. at 37°C. were examined for interfacial precipitate. The results obtained in this way were confirmed by complement fixation tests.

Precipitin reactions in gels.

The method used is described by Oudin (1952),

a mixture of agar and BS07 immune serum being used as internal reactant, the external reactants being solutions of the purified polysaccharides.

Cultures of the organisms

(a) For Fuller extraction.

1 ml. of an 18 hr. culture was inoculated into 4 l. of medium containing 1% Lab-lemco (Oxo Ltd., London), 1% peptone (Evans Medical Supplies Ltd., London), 0.5% NaCl (A.R.), 0.5% glucose (A.R.) which was incubated at 40°C. for 48 hr. The medium, with the exception of the glucose was sterilised by autoclaving for 15 min. at 121°, and the glucose, sterilised by filtration, added as a 50% solution after cooling. The initial $_{p}$H of the medium was 6.8.

(b) For Lancefield extraction

1 ml. of an 18 hr. culture was inoculated into 4 l. of medium containing 1% peptone, 0.5% yeast extract (Difco dehydrated. Difco Co., Detroit, Michigan, U.S.A.), 0.5% Lab-lemco, 0.5% Bacto-tryptone (Difco), 0.5% glucose, and incubated for 48 hr. at 40°. The medium was sterilised as before, the initial $_{p}$H being 7.6

(c) For preparation of polysaccharides from the metabolic liquid and capsules.

The medium used was as in (a).

Preparation of the polysaccharides.

Fuller extraction.

The procedure used was similar to that described by Fuller (1938) but as larger quantities of cells were used it was modified as follows. The cells were removed from a 48 hr. culture by centrifugation on a Sharples Supercentrifuge (Sharples Centrifuge Co., Stroud, Glos.) and after washing twice with sterile water on an angle centrifuge the sediment was suspended in formamide (B.D.H. Laboratory Reagent; 40 ml.) and heated at 150°C. in an oil bath for 15 min. After cooling, acid-alcohol (100 ml. containing 95 ml. ethanol, 4 ml. water, 1 ml. conc. HCl) was added with stirring and the precipitate removed on the centrifuge. To the supernatant liquid, acetone (200 ml.) was added and the precipitate centrifuged off after 10 min., dried by trituration with acetone and finally in a vacuum desiccator over P_2O_5.

Lancefield extraction.

The washed cells were prepared from 4 l. of a 48 hr. culture as for the formamide extraction. The organisms were suspended in 0.05N-HCl(80 ml.) containing a few drops of Congo-red (0.2% aqueous solution). 1N-HCl was then added until the

indicator turned slate blue. The whole was then immersed in a boiling water bath for 10 min., when a pink colour developed, and after rapid cooling, centrifuged. The sediment was discarded. After addition of a few drops of phenol red (B.D.H. Indicator) the clear supernatant was neutralised with 1N-NaOH and recentrifuged. The supernatant solution is usually used for precipitin tests without further treatment. However, in order to obtain a solid for analysis the polysaccharide was precipitated by the addition of acetone (2 vols.) and dried in acetone <u>in vacuo</u> over P_2O_5. Precipitation of the material did not appear to alter the serological reactions. For precipitin tests a known amount of the material was dissolved as far as possible in physiological saline (0.85% w/v NaCl) and any insoluble residue centrifuged off, dried and weighed, to give the amount of material in solution.

The results of analyses and precipitin reactions of both extracts are shown in Tables 13 and 14. The sugars found in the hydrolysates were the same in each extract and amino acids in a hydrochloric acid hydrolysate showed the presence of protein in the original material. While the formamide extract gave weak reactions with group-D

Table 13. The analysis of serologically active materials from an amylolytic rumen streptococcus BS07

Preparation	Yield ⁂ (mg.)	Sugars (as glucose) after hydrolysis ø %	Ash %	N %	Uronic acid %	Rhamnose %	Sugars in hydrolysate ⊖
Fuller (formamide) extract.	101.1	34.9	-	8.6	+	-	glucose, galactose, rhamnose, ribose.
Lancefield (acid) extract	309.5	41.0	-	4.3	+	13.5	glucose, galactose, rhamnose, ribose, hexosamine.
Metabolic liquid polysaccharide (18 hr. culture).	184.0	37.4	-	3.8	+	4.4	glucose, galactose, rhamnose, ribose.
Metabolic liquid polysaccharide (48 hr. culture).	1403	11.2	74.7	0.6	6.3	1.5	glucose, galactose, rhamnose, xylose.
Capsular poly-saccharide (48 hr. culture).	113.4	58.2	7.2	0.7	22.0	17.6	galactose, rhamnose; glucose (trace).

Phosphorus content of capsular polysaccharide 0.2%

ø 6 hr., 1.5N-H_2SO_4 at 100°. 3 hr. hydrolysis gave slightly lower results. (corrected for destruction of sugars by acid).

⊖ Identified by paper chromatography.

⁂ Yield, mg./5 ml. culture.

\+ Qualitative test showed positive result.

Table 14. Precipitin titres of serologically active materials from amylolytic rumen of streptococcus BS07

Preparation	Precipitin titre (mg./ml.x 10^5) ø BS07 immune serum	Group D serum
Fuller (formamide) extract	1,200	12,000 (or rather less)
Lancefield (acid) extract.	120.6	no reaction
Metabolic liquid polysaccharide (18 hr. culture).	71.0	55,800
Metabolic liquid polysaccharide (48 hr. culture).	1404 (800*)	10,800
Capsular polysaccharide (48 hr. culture).	17.7 (13.0*)	453,000

ø Least weight of preparation to give a positive precipitin.

* Figures obtained using a complement fixation titration. The lower values suggest that this method is more sensitive than the precipitin reaction.

serum no reaction could be detected with the acid extract. Both showed positive reactions with the homologous immune serum, but while the acid extract did not react with homologous serum after the solution had been stored at room temperature for 48 hr., the reaction of the formamide extract was unaltered after a similar period.

Schmidt (1952) in his studies of group A streptococcal polysaccharide digested the protein in the cells with pepsin. An attempt was made to remove the protein from an acid extract prepared from Streptococcus faecalis (N.C.T.C. 775) by digestion with crystalline trypsin (Armour Laboratories, Lindsay St., London E.C.1.), but analysis of the resulting material showed no improvement in the nitrogen, reducing sugar content (after hydrolysis) or precipitin titre over the untouched extract, so the method was not further investigated.

Preparation of a polysaccharide from the metabolic liquid.

Since it was possible to demonstrate the presence of a serologically active material in the metabolic liquid of the streptococci (Section 2), it was thought that a polysaccharide uncontaminated by intracellular material might easily be obtained

from this. The method used was a modification of that of Kendall et. al. (1937) whose method has also been used by Loewenthal (1938) for the isolation of a polysaccharide from haemolytic streptococci. A young culture was used as this would be less likely to contain material from autolysed cells.

The cells were removed from 1 l. of an 18 hr. culture (see above) by centrifuging at + 1°C. (all subsequent operations were carried out at + 1°C.). The sediment was washed with two lots of 0.1M-phosphate buffer ($_pH$ 7.0; total 130 ml.) and the washings added to the original supernatant. To the clear liquid (approx. 1 l.) alcohol (2 vols.) was added and the whole stored at + 1°C. for 48 hr. The precipitate was removed on the centrifuge and dissolved in water (200 ml.) containing hydrated sodium acetate (10 g.) and glacial acetic acid (5 ml.). The solution was divided into two portions and deproteinised by repeated shaking with chloroform (25 ml.) and butanol (2.5 ml.) (Sevag, 1934). The final aqueous solution was recentrifuged and to the clear liquid two volumes of alcohol were added. After storing for 18 hr. the fine precipitate was removed on the centrifuge, dissolved in water, diluted to 50 ml. and centrifuged. A portion (1 ml.) was removed for precipitin tests,

and the polysaccharide quantitatively precipitated from the remainder of the supernatant by addition of alcohol (2 vols.) and dried in acetone as before. The results of analysis and the precipitin titrations are given in Tables 12 and 13.

Preparation of the capsule polysaccharide.

Morgan (1937) described several organic solvents which would dissolve polysaccharide from a number of organisms. Of these ethylene and diethylene glycol appeared to be the best. Seastone (1939) removed the capsules from Group C haemolytic streptococci with phosphate buffer, $_{p}$H7, and with 3% aqueous chloroform. All these reagents were tried on small portions of washed cells of streptococcus BS07. Wet Indian ink preparations showed no reduction in capsule size after treatment and no precipitates were formed on addition of excess alcohol to the supernatant liquids. The streptococcal cells were then heated in acetate buffers of different $_{p}$H values and it was found that a few minutes heating at 100°C. in a buffer of $_{p}$H removed the capsules without apparently damaging the cells (Figs. 18 and 19). This method is similar to that of Porges (1905) who used hot 0.25 N-HCl for removing the capsules of Klebsiella pneumoniae. This method was adopted for the large

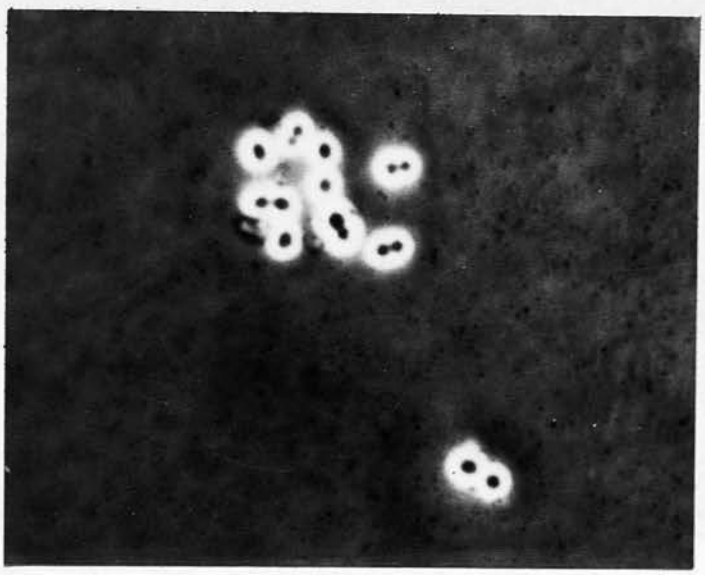

Fig. 18. Pure culture of rumen streptococcus BS07 with capsules outlined by Indian ink particles.
Wet preparation, phase contrast X 2,400.

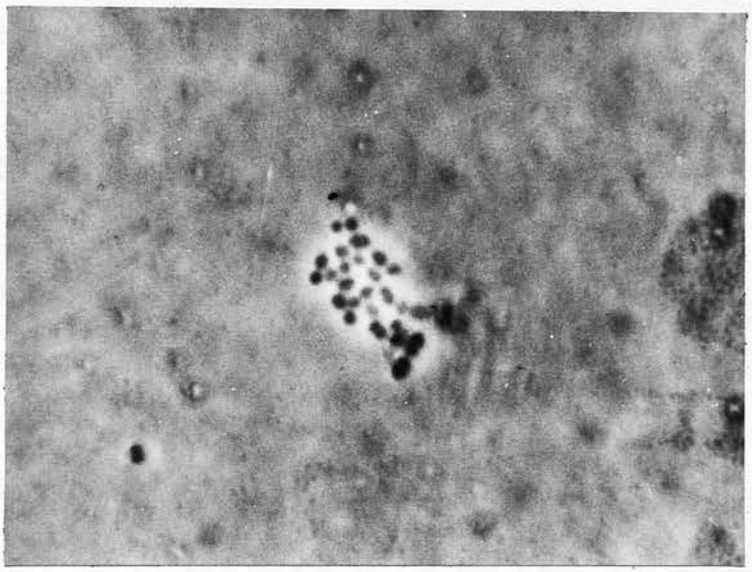

Fig. 19. The same preparation as in preceding Figure after removal of capsules with acetate buffer, $_{p}H$ 5.2.
Wet Indian ink preparation,
phase contrast X 2,400.

scale preparation of the capsule polysaccharide.

The cells were removed from 5 l. of a 48 hr. culture on the Sharples Supercentrifuge and washed three times with distilled water on an angle centrifuge. To the wet suspension of cells (100 ml., concentration of organisms approx. 10^{10}/ml.) 0.2M-sodium acetate ($_{p}$H adjusted to approx. 2 with glacial acetic acid; 100 ml.) and Tween 80 (Honeywell and Stein Ltd., London; 0.2 ml.) were added, and the whole heated in a boiling water bath with frequent shaking. At intervals small samples were removed, neutralised with 5N-NaOH and examined in wet Indian ink preparations. The decrease in size of the capsules on heating is shown in Table 15.

After 25 min. the majority of the cells were decapsulated, their appearance before and after acid treatment being shown in Figs. 18 and 19. The suspensions were rapidly cooled, centrifuged, and the supernatant washed with water. The supernatant and washings (300 ml.) were divided into two portions and deproteinised by shaking with chloroform and butanol as above. To the final aqueous layer, alcohol (2 vols.) was added and after storing at + 1°C. for 48 hr. the precipitate was removed on the centrifuge and dissolved in water

Table 15. Removal of capsules from amylolytic rumen streptococcus BS07 with sodium acetate/acetic acid, $_{p}$H 2.

Time of heating in 100° water bath (min.)	Overall diameter of cell and capsule (μ) ⁋
0	2.1
10	2.1
20	1.4
25	1.0 ∅

∅ Cells not visibly damaged

⁋ Measured with calibrated micrometer eyepiece across shortest diameter.

(50 ml.) to give a clear viscous solution. This was centrifuged, a portion removed for precipitin tests and the polysaccharide quantitatively precipitated from the remainder by addition of a little NaCl and alcohol (150 ml.). The polysaccharide was dried by trituration with acetone and in a vacuum desiccator over P_2O_5, to give a white powder entirely soluble in water to a clear viscous solution.

A polysaccharide was prepared by the method previously described after filtering and concentrating the metabolic liquid (5 l.) to approx. 650 ml. under reduced pressure. The qualitative analysis of this material was similar to that previously prepared from the 18 hr. culture, but it contained a very large amount of ash. This was probably mainly caused by evaporation of the liquid to a small volume before the first precipitation.

The results of the analysis and precipitin tests are shown in Tables 13 and 14. Precipitin reactions were also carried out in agar-serum gels, the external reactants being the capsular polysaccharide (4.5 mg./ml. in water) and the polysaccharide from the metabolic liquid (13.6 mg./ml.) (Fig. 20). The capsular polysaccharide diffused furthest into the gel forming a single band of precipitate with a sharp leading edge and a rather

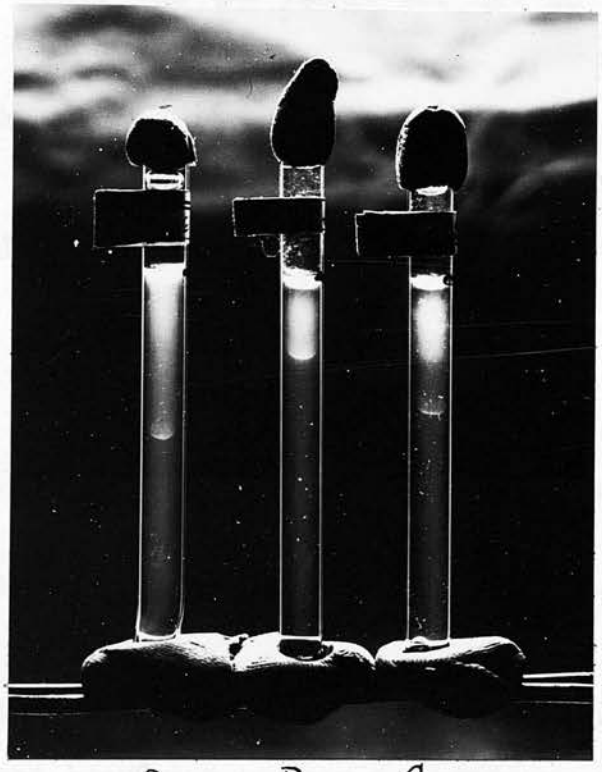

Fig. 20. Precipitin reactions in gels.
The internal reactant (antiserum) was incorporated in 0.6% agar. The external reactants were solutions of the purified polysaccharides.

A ... capsular polysaccharide
B ... metabolic liquid polysaccharide
C ... mixture of A and B.

diffuse upper part. The polysaccharide from the metabolic liquid formed a diffuse band which travelled about half the distance of the capsular polysaccharide, and the front was not so well defined. Using a mixture of the two polysaccharides as external reactant two precipitate bands were formed in the gel, the distances travelled corresponding with those of the separate solutions.

DISCUSSION

Two methods are in general use for serological classification of streptococci. These are based on group- and type-specificity. In Lancefield's classification (Lancefield, 1933) streptococci having a common antigen are placed in the same group, designated by one of the letters A to N. Intestinal streptococci have, in general, been found to belong to group D. Groups A and D streptococci have been subdivided into types by Griffiths (1935) and Sharpe and Shattock (1952) respectively. These types possess, besides the common group antigen, a type specific antigen which has been associated with polysaccharide in the capsule (Kendall *et. al.* 1937). The group specific substance has been shown to be intracellular and to

be either protein or polysaccharide in nature (Heidelberger and Kendall, 1936).

In the experiments described, the Lancefield and Fuller methods, although intended to extract similar antigens, gave products with different serological reactions (Table 14). The great difference between the reagents, and the somewhat drastic procedures involved in the extractions would be possible reasons for the discrepancies in the reactions of the products.

The polysaccharide isolated from the metabolic liquid of an 18 hr. culture had a lower nitrogen content than the acid and formamide extracts and a higher precipitin titre with the homologous type serum, but, in high concentration, it gave a reaction with the group serum. This was to some extent non-specific, as solutions of higher concentration gave a reaction with normal rabbit serum. The capsular polysaccharide reacted in extremely low concentration (17.7×10^{-5} mg./ml.) with the homologous type serum and only in high concentration (4.5 mg./ml.) gave a faint reaction with the group D specific serum. No reaction was given by the polysaccharide in this concentration with normal serum. No conclusions can be drawn about the group specific material in the organisms

except to suggest that it is probably intracellular as it is found in the formamide extract of whole cells and in a greater amount in the metabolic liquid polysaccharide of the old culture (48 hr.) than the 18 hr. culture, and the former would be expected to contain more autolysed cells. The injection of capsular polysaccharide into rabbits to see if it acts as a complete antigen has not been tried.

The results of the precipitin reactions in gels (Fig. 20) suggest that the metabolic liquid polysaccharide is of somewhat higher molecular weight than the capsular polysaccharide, or is linked to protein not present in the capsular polysaccharide, as it diffused a smaller distance into the gel. As each solution formed only one precipitate band it may be assumed that there is only one constituent in each preparation capable of giving a precipitin reaction with the BS07 immune serum. This is supported by the results of ultracentrifuge examination, reported in the Addendum, which show that the capsular polysaccharide is essentially homogeneous in molecular size.

Galactose, rhamnose and uronic acid were common to the sugar constituents of the hydrolysates of the preparations. Glucose was also present in a

similar concentration in the hydrolysates of the acid and formamide extracts and the two polysaccharides isolated from the metabolic liquids. This glucose may be present in an intracellular polysaccharide (it has been found in hydrolysates of whole group A streptococci by Consden and Stanier, 1952), and the metabolic liquid might be expected to contain material from some autolysed cells, or it may be due to some extent to adsorption from the medium onto the precipitates. The latter is possible as traces of arabinose were found in metabolic liquid polysaccharide hydrolysates and control experiments showed that small amounts of this sugar were present in the medium together with the glucose. The small amount of xylose in the metabolic liquid (48 hr. culture) polysaccharide is at present unexplained, although it has been found in some bacteria (Behorzerski, 1952; Hofmann, 1953). The presence of ribose in the acid and formamide extracts and traces in the metabolic liquid (18 hrs.culture) polysaccharide suggest that nuclear material is contained in these preparations. No ketose sugars were found in hydrolysates, but some hexosamine could be detected in chromatograms of the hydrolysate of the acid extracted polysaccharide. The purified capsular polysaccharide is composed of galactose,

rhamnose, a uronic acid, probably galacturonic acid, with a trace of glucose. Like some other polysaccharides containing uronic acids (see for example, Hough, Jones and Wadman, 1952) the initial rate of acid hydrolysis was rapid, but after 3 hr. in 1.5N-H_2SO_4 only a slow increase in reducing sugars with time of heating was found (after correcting for the slight destruction of the sugars by the acid). This was not increased by the use of 3N-acid, which greatly increases the rate of destruction of the sugars. Galactose, rhamnose and galacturonic acid have a lower reducing power than glucose with the reagents used, and this and the slow rate of hydrolysis would account for the apparent low reducing sugar content after hydrolysis.

A purification of the type specific material from this streptococcus had thus been achieved and it has been shown to be a capsular polysaccharide in conformity with the results obtained with other bacteria. Schmidt obtained a polysaccharide from group A streptococci containing 26.1% glucosamine, 60.8% rhamnose and 2.6% nitrogen which reacted in high dilution with group A serum. Consden and Stanier (1952) reported glucose, rhamnose, uronic acid and hexosamine in unpurified hydrolysates of group A streptococci. Hofmann (1953) found galactose,

ribose, rhamnose and xylose in hydrolysates of nitrosomonas. Polysaccharides containing uronic acids have been isolated from the pneumococci and the polysaccharide from this amylolytic streptococcus resembles that of Type II pneumococcus (Kent, 1952) which contains glucose, rhamnose, and glucuronic acid.

SUMMARY

(1) Materials extracted from an amylolytic rumen streptococcus by the Lancefield and Fuller methods have been compared for serological activity and chemical composition. Similar tests have been made on polysaccharides prepared from the metabolic liquid.

(2) A method has been developed for removing the capsules from these streptococci.

(3) The capsular polysaccharide has been shown to posess a high type-specific serological activity.

(4) An analysis of the capsular polysaccharide has been carried out and its main constituents have been shown to be galactose, rhamnose and a uronic acid, the analysis being; reducing sugars, as glucose, after acid hydrolysis, 58.2%, N 0.7%, P. 0.2%, ash 7.2%, uronic acid 22.0%, rhamnose 17.6%

ADDENDUM TO SECTION 4

The purified capsular polysaccharide was examined in the ultracentrifuge and its intrinsic viscosity in solution was measured by Dr. C. T. Greenwood (Chemistry Department, University of Edinburgh).

Dr. Greenwood found :-

(1) that the polysaccharide was homogeneous and only moderately disperse.

(2) that the material had a high intrinsic viscosity and hence that the molecules were very asymetrical.

(3) that the molecular weight of the polysaccharide, calculated from sedimentation data and viscosity measurements, was 90,000.

Few measurements have been made on similar polysaccharides from other bacteria, but Record and Stacey (1948) have reported molecular weights of 171,500 and 141,000 for pneumococcus types I, II and III polysaccharides.

SECTION 5

THE AMYLASES OF THE STREPTOCOCCUS BS07 AND A STRAIN OF CLOSTRIDIUM BUTYRICUM ISOLATED FROM THE RUMEN OF THE SHEEP

In the course of an investigation into the digestion of starch by the pig, Baker, Nasr, Morrice and Bruce (1951) isolated a micro-organism responsible for the breakdown of starch in the caecum and identified it as a strain of Clostridium butyricum. The extra-cellular amylase formed when this strain of Cl. butyricum was grown on a medium containing soluble starch was examined by Whelan and Nasr (1951) and was shown to be of the α-amylase type.

Masson (1951) isolated another strain of Cl. butyricum which was concerned in starch breakdown in the rumen of sheep fed on a flaked maize and hay diet. Both this clostridium and the streptococcus whose isolation from sheep rumen was described in Section I were shown to form an extra-cellular amylase when grown in a medium containing dissolved starch. It was decided, in collaboration with Dr. P. N. Hobson, to investigate these amylases. In this section, there is presented a detailed description of the bacteriological techniques and

simplified account of the biochemical methods which were employed. A short account of the results and a discussion of their relation to other bacterial amylases hitherto investigated is also given.

The amylases.

Starch consists of a mixture of two mucin components, amylose (25%) and amylopectin (75%). Amylose consists mainly of straight chains of glucose units and gives a pure blue coloration with iodine. Amylopectin consists of short chains of glucose units which are extensively branched and gives a purple colour with iodine.

The amylases have been classified into two main types known as α - and β - amylases. Naturally occurring amylases may be of one or other of these types, or contain a mixture of both.

Both amylases hydrolyse amylose to maltose but they differ in their behaviour towards amylopectin. Neither of them can hydrolyse the branching links of the amylopectin molecule but α - amylase hydrolyses its other linkages almost completely so that dextrins having only a very low molecular weight and giving no colour with iodine are left. β - Amylase, on the other hand, breaks down amylopectin much less extensively, forming β - dextrins which still give a portwine colour with iodine.

The bacterial amylases so far studied have been of the α - type. They include :- the amylose of B. subtilis investigated by Hopins and Kulka (1942) and Hopkins, Dolby and Stopher (1942) and by Di Carlo and Redfern (1947 and 1948); the amylase formed by B. polymyxa (Rose, 1948); the amylase of Cl. acetobutylicum (Hockenhull and Herbert, 1945); and that formed by Cl. butyricum from pig caecum investigated by Whelan and Nasr (1951).

METHODS AND MATERIALS

Amylose and Amylopectin were obtained by the fractionation of potato starch by the method of Hobson, Pirt, Whelan and Peat (1951).

β - Dextrin was obtained by the hydrolysis of amylopectin with soyz-bean β - amylase as described by Hobson, Whelan and Peat (1950).

α - Amylase. The amylase of human saliva is of the α - type. Saliva was collected as needed, diluted with an equal volume of water and centrifuged to remove mucin. The supernatant was diluted to the required activity.

β - Amylase. Soya-bean amylase was used as source of amylase of the β - type (cf. Hobson et. al. loc. cit.)

Measurement of amylase activity.

A colorimetric method was used which depends on the

following principle :- The blue colour produced by adding a standard amount of a starch preparation (amylose or β - dextrin) to iodine is compared with that given by the same amount of starch after incubation with the amylase under standard conditions. The reduction of the blue colour (due to enzymatic hydrolysis of some of the starch) gives a measure of the activity of the enzyme.

The "test" digest mixture contained 3.75 ml. amylose (1 mg./ml.), 1.5 ml. sodium citrate buffer (0.2 M, $_{p}$H 7.0) and 1.75 ml. enzyme solution. It was incubated at 35°C. for 30 min. One ml. was removed and stained with iodine (2 mg.) KI (20 mg.)/ 100 ml. water. A "blank" digest containing water in place of enzyme solution was simultaneously incubated and its blue iodine colour was developed in the same way. The absorption of light (wavelength 680 mμ.) by these blue solutions was measured photometrically in an EEL colorimeter. The difference in the extinction values (E.V.) of the "test" hydrolysate compared with that of the "blank" was strictly proportional to the amount of enzyme in the "test" digest mixture. One unit of amylase activity was defined as the amount of amylase which produced a fall in the E.V. of one colorimeter scale unit under the defined conditions of assay.

Determination of reducing sugars.

The copper reagent of Somogyi (1945) was standardised against maltose and the reducing power of the digests was estimated iodometrically.

Preparation of cell-free filtrates.

(1) Cl. butyricum. Cultures of the clostridium were inoculated into a modified Beijerinck fluid medium (400 ml.) containing 5% soluble starch (Baker et al.1951) and incubated at 40° for 40 hr. After this time all iodine-staining polysaccharide in the medium had been degraded and gas production had almost ceased. The culture was centrifuged for 30 min. at 740 g. to remove most of the cells, and then passed through a Seitz filter fitted with a Carlson 'EK' sterilizing pad. The volume of sterile filtrate obtained was usually about 300 ml.

(2) Streptococcus. The medium used for these organisms was a yeast broth (200 ml.; 1% 'Difco' yeast extract, 1% Bacto Tryptone, 0.1% KH_2PO_4, pH adjusted to 6.8) containing 5% dissolved starch and 3% (w/v) $CaCO_3$. The culture was incubated at 40° for 40 hr. with occasional shaking to suspend the $CaCO_3$. At this time amylase activity was maximal. The culture was then centrifuged until clear (20 min. at 1300 g) and sterilized by filtration as above.

The volume of filtrate obtained was about 150 ml.

Preparation of freeze-dried enzymes.

Preliminary experiments were carried out to determine the optimum concentrations of $(NH_4)_2SO_4$ for precipitation of the enzymes. All precipitations were carried out as near as possible to + 1°. Freeze drying was carried out in 1 l. round-bottomed flasks at 0.1 mm. pressure using an acetone and solid carbon dioxide condenser. (1) Cl. butyricum. The precipitation of the enzyme from the culture filtrate was carried out by addition of ammonium sulphate to a concentration of 40% (w/v) and the precipitate was freeze-dried. It was then dissolved in water and re-precipitated by addition of ammonium sulphate to a concentration of 30% (w/v). After standing for 90 min. this precipitate was centrifuged, washed with 30% ammonium sulphate, dissolved in 0.02M sodium citrate buffer ($_pH$ 7.0) and freeze-dried. The initial activity in the culture filtrate was 19,800 units. 17,000 units were recovered in the final dried precipitate (weight 0.4609 g.).
(2) Streptococcus. Ammonium sulphate was added to the culture filtrate to a concentration of 40% (w/v). After standing for 14.5 hr. at + 1°, the precipitate was centrifuged, washed and dissolved in water. It was re-precipitated by adding ammonium sulphate to

a concentration of 35% (w/v) and, after standing for 4.5 hr., was centrifuged, washed with 35% ammonium sulphate, dissolved in 0.02M sodium citrate buffer ($_pH$ 7.0) and freeze-dried. About half of the activity in the original filtrate was recovered in this final preparation (activity 13.140 units, weight 0.6923 g.).

These freeze-dried powders kept their activity indefinitely when stored at + 1°C.

RESULTS

The type of amylase present.

This was decided by testing the activity of the enzyme using (1) amylose and (2) β - dextrin as substrate in the standard test digests. Since a β - amylase will abolish the iodine-colour of amylose but will have no effect on the iodine-colour of β - dextrin, whereas an α - amylase will abolish both these colour reactions, curves characteristic of the two types of enzyme can be obtained by plotting the extinction values obtained after allowing equal amounts of enzyme to act on each of these substrates.

Standard digests, both containing the same enzyme preparation, but one containing amylose and the other β - dextrin, were incubated together and,

at equal periods after the addition of the enzyme, portions were removed for determination of the E.V. The bacterial enzyme preparations were compared under these conditions with soya-bean β - amylase and human salivary α - amylase. Fig. 21 shows that the curves given by both the bacterial enzymes resembled very closely that given by salivary α - amylase and were quite distinct from the typical β - amylase curve of the soya-bean enzyme, which has no action at all on β - dextrin.

Optimum $_pH$ and temperature of action of the enzymes.

The optimum $_pH$ of action was measured by determining the fall in EV (680 mμ.) of digests like the standard-activity digests but containing a sodium acetate-sodium veronal buffer the $_pH$ of which had been adjusted by the addition of 0.1N-H_2SO_4 (5 ml. 0.143M-sodium acetate-sodium veronal solution, x ml. acid, (19 - x) ml. water). The constituents were preheated to 35°, mixed, and the EV (680 mμ.) of portions of the digests were measured after incubation for 15 and 30 min. as in the determination of activity. The $_pH$ values of the digests were checked at the end of the incubation.

Both enzymes have a broad $_pH$ optimum. That of the streptococcal enzyme under these conditions lies between 5.5 and 6.5 and that of the clostridial

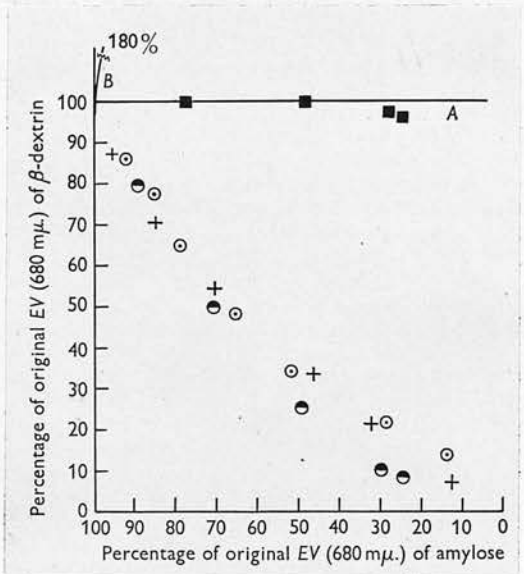

Fig. 21. The comparative action of the enzymes on amylose and β - dextrin.
Soya-bean β - amylase (■), clostridial amylase (+), salivary amylase (◓), at $_{p}$H 7.0. Streptococcal amylase (⊙), at $_{p}$H 6.0. All digests incubated at 35°.

enzyme between 4.6 and 6.1 (Fig. 22). This latter value is similar to the optimum $_{p}H$ range of crystalline B. subtilis enzyme (5.3 - 6.8; Meyer et al.1947), partially purified B. subtilis amylase (5 - 6.5; Di Carlo and Redfern, 1947) and B. polymyxa amylase (6.2 - 7.5; Rose, 1948).

The optimum temperature was determined by incubating the standard activity digests at different temperatures and determining the fall in EV (680 mu.) after 15 and 30 min. in the usual manner.

The results in Fig. 23 show that both enzymes have the same optimum temperature of 48 $\pm$ 1° under the digest conditions employed. For subsequent experiments a temperature of action of 35° was chosen for both enzymes.

Tests for the presence of other enzymes.

No maltase, phosphorylase, phosphatase or invertase activities could be demonstrated in these partially purified preparations of bacterial amylase.

The hydrolysis of starch and starch fractions by the enzymes.

Solutions of the amylose, and amylopectin from potato starch were prepared and incorporated in digests of the standard proportions containing buffers of $_{p}H$ values 4.8 and 6.0 for the clostridial amylase and 6.0 for the streptococcal amylase. The

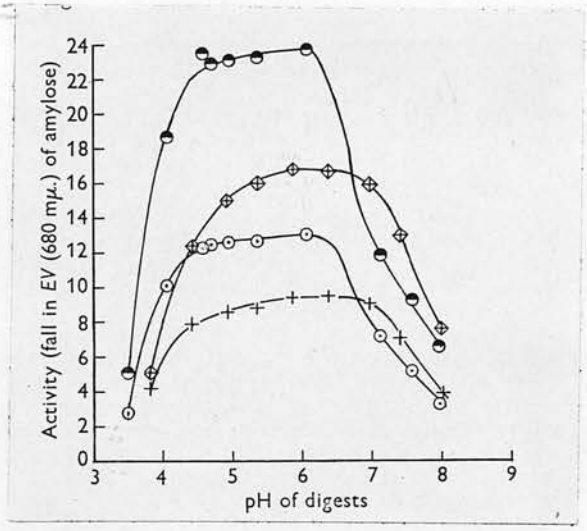

Fig. 22. The effect of pH on amylase activity at 35°. Incubation periods: clostridial amylase, 15 min. (⊙), 30 min. (◓); streptococcal amylase, 15 min. (+), 30 min. (⊕).

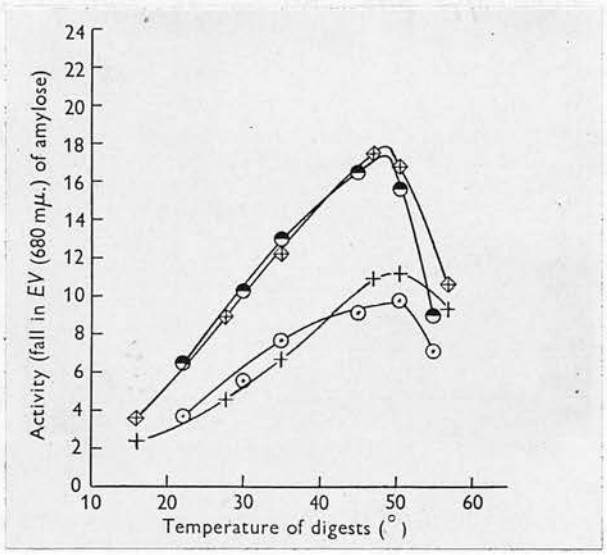

Fig. 23. The effect of temperature on amylase activity.
Digests at $_{p}$H 7.0. Incubation periods: clostridial amylase, 15 min. (⊙), 30 min. (◓); streptococcal amylase, 15 min. (+), 30 min. (⊕).

digests were incubated at 35°, and, at intervals, the reducing sugars present were determined and expressed as maltose. The activities of the enzymes in the digests were determined at intervals.

After 27 hr. the streptococcal enzyme retained its initial activity in the digests (4.7 units/1 ml.), but at 72 hr. the activity had fallen to 2.9 units/1 ml., and at 118 hr. it was 0.8 unit/1 ml. The clostridial amylase retained its full activity at 24 hr., and the activity at 118 hr. was 1 unit/1 ml. At 72 hr. fresh enzyme was added to portions of each digest containing amylose and, to the digests containing the clostridial amylase, starch and amylopectin to bring the activity to the initial value; the extents of conversion to reducing sugars did not, however, markedly increase above those of the original digests. The apparent conversions to maltose of the different substrates by the two enzyme preparations are shown in Figs. 24 and 25; in each case the values found for starch are almost exactly those calculated from the extent of the hydrolysis of the two constituents, amylose and amylopectin.

<u>The significance of amylase in the metabolism of Streptococcus BS07 and Clostridium butyricum</u>.

Identification of the main products of the

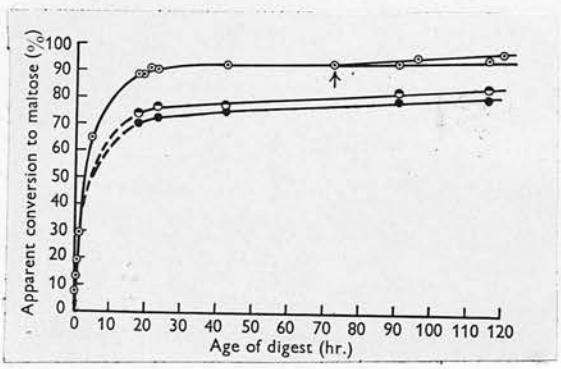

Fig. 24. The hydrolysis of starch fractions by the streptococcal amylase at 35°.
Digests at $_{p}$H 6.0; amylose (⊙), starch (◓), amylopectin (●). Addition of enzyme to bring the activity to the initial value (↑).

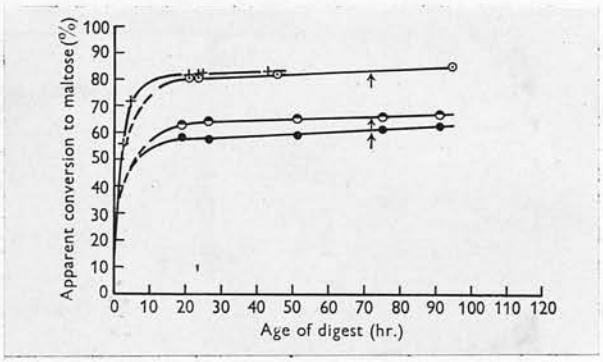

Fig. 25. The hydrolysis of starch fractions by the clostridial amylase at 35°.
Digests at $_{p}H$ 4.8; amylose (⊙), starch (◓), amylopectin (●). Digest at $_{p}H$ 6.0; Amylose (+). Addition of enzyme to bring the activity to the initial value (↑).

hydrolysis of amylose by these bacterial enzyme preparations was carried out by chromatography on filter paper and charcoal. Glucose, maltose, maltotriose and maltotetraose were identified as the end-products of their activity.

In other experiments it was found that both organisms grew on media containing glucose, maltose or maltotriose as the only source of carbohydrate and that acids were produced by the fermentation of these sugars. Thus these organisms can use as energy sources the main products of the hydrolysis of starch by their isolated extra-cellular amylases.

The amylases would appear to be constitutive and not adaptive enzymes since, when the clostridium was grown in a medium containing maltose as the only carbohydrate, and the streptococcus on a glucose-containing medium, the other constituents being the same as in the starch medium, amylase activity was present in the cell-free filtrates.

SUMMARY

(1) Starch-degrading enzymes were isolated in the form of stable freeze-dried powders from cell-free filtrates of cultures of sheep rumen strains of Clostridium butyricum and a

a streptococcus grown in media containing dissolved starch. Their properties were examined.

(2) The enzymes belonged to the group of α - amylases. The hydrolysis of starch and starch fractions by the enzymes was investigated. The branch linkages in amylopectin acted as obstructions to both enzymes.

(3) Maltose and maltotriose with some glucose were the main end products of the hydrolysis of amylose by these enzymes.

(4) The organisms could utilise the end-products of starch hydrolysis as energy sources.

(5) The amylases of these organisms were constitutive and not adaptive enzymes.

GENERAL DISCUSSION

It is only in recent years that the physiology of ruminants has been intensively studied by modern techniques and some knowledge gained of the end-products of ruminant digestion.

The complexity of the rumen micro-flora and of the biochemical reactions ascribed to it were described in the Introduction. The main impression gained from a review of the information available is that much of the work has been, from a bacteriological point of view, inadequate. A different approach is required for the study of the complex mixed culture of interdependent symbionts which inhabit the rumen from that used in the study of pathogenic bacteria by the methods of classical bacteriology.

Purely morphological studies, though necessary in preliminary surveys, are of limited value due to the extreme pleomorphism exhibited by many rumen organisms. Useful observations can, however, be made by systematic microscopic examination, especially if this is supplemented, as was shown in the present study, by the use of phase contrast and by histochemical staining reactions such as the iodine test for iodophilic organisms.

The use of counting techniques in the study of

the rumen is limited in scope. The value of counting methods has frequently been over-emphasised. They measure only those organisms which are free in the rumen liquid and take no account of those fixed on or even in food particles - the cellulose digesters for example - whose importance cannot be denied although counting these is virtually impossible. Gall and Huhtanen excluded facultative anaerobes from the "true" rumen bacteria because they were present in a concentration of less than 1×10^6/g. of rumen contents. Such a conclusion seems too arbitrary since organisms vary greatly in the ease with which they can be cultured. The numbers of an organism obtained in a viable count can not be made the sole criterion of its physiologal importance.

Other difficulties arise when an attempt is made to use pure cultures of rumen bacteria in the study of the metabolism of the rumen. The food of the ruminant is heavily contaminated with bacteria and it is necessary, therefore, to be able to distinguish between these organisms and "true" rumen bacteria. Gall and Huhtanen suggested cultural criteria with which "true" rumen organisms ought to comply but their postulates are open to considerable criticism. Thus, although the rumen may be essentially an aerobic organ, air is frequently

introduced into it by swallowing and may be sufficient in amount to support a small proportion of aerobes in the rumen population. The conclusions of Gall and Huhtanen as to what constitutes a "true" rumen organism may also be criticised on technical grounds. Their equation of obligate anaerobiosis with a clear zone between the surface of the agar and the beginning of growth is not always borne out by their other results. Some of their "obligate anaerobes" may well be facultative anaerobes. The amylolytic streptococci isolated in the present study and unequivocally shown to be present in the rumen by the capsule swelling reaction were, in fact, facultative anaerobes. A method, such as the simple serological test described in Section 2, which allows the demonstration of the cultured organisms <u>in situ</u>, is a more convincing proof that an organism is a true rumen bacterium than arbitrary criteria based on counting methods or cultural characteristics.

A further difficulty which may arise in pure culture studies was brought to light by the work of Sjipesteijn who showed that cellulose digesters, which grew only very slowly in pure culture <u>in vitro</u>, would grow and digest cellulose much more rapidly in the presence of other organisms. The interdependence of the rumen micro-flora may thus create a problem

which is most difficult to study by pure culture techniques *in vitro*.

Having isolated a "true" rumen organism in pure culture, there still remains the problem of its function in the economy of the host animal. The study of the amylase of the streptococci, whose isolation was described here, was relatively simple because the biochemical techniques required were already available. The study of cellulose breakdown is a very different matter, for knowledge of the enzymes involved in this process is only slight.

Lastly, the possible importance for the host of synthetic activity by rumen bacteria must be considered. The work which has already been done on the nutritional value of bacterial protein and on the synthesis of bacterial protein from non-protein nitrogen was reviewed in the Introduction. It was shown in the present study that amylolytic streptococci form large amounts of capsular material in the rumen. This material was shown to be a polysaccharide which may perhaps be of nutritional value to the host at a later stage in the digestive cycle.

There is a vast field here for future research which can probably only be fully explored by the use of isotope techniques.

REFERENCES

Allen, R.J.L. (1940) Biochem. J., 34, 859.

Bacon, J.S.D. and Edelman, J. (1951) Biochem. J., 48, 114.

Baker, F. and Martin, R. (1938) Nature, Lond., 141, 877.

Baker, F. (1941) Nature, Lond., 149, 220.

Baker, F. (1942) Nature, Lond., 150, 479.

Baker, F. and Harris, S.T. (1947) Nutr. Abstr. Rev. 17, 3.

Baker, F. and Nasr. H. (1947) J. R. micr. Soc., 67, 27.

Baker, F., Nasr, H., Morrice, F., Bruce, J. (1951) J. Path. Bact., 62, 617.

Bélozerski, A.N. (1952) 2nd. Int. Congr. Biochem., Paris.

Breed, R.S., Murray, E.G.D., and Hitchens, A.P. (1948) Bergey's Manual of Determinative Bact., London, 6th. ed.

Brewer, J.H. (1942) Science, 95, 587.

Bortree, A.L., Smith, C.K., Sarker, B.C., Huffman, C.F. (1948) J. Anim. Sci., 7, 520.

Bryant, M.P. (1952) J. Bact., 64, 325.

Carter, C.R. (1952) Canad. J. med. Sci., 30, 48.

Cole, H.H., and Kleiber, M. (1948) J. Dairy Sci., 31, 1016.

Colebrook, D.C. (1935) Spec. Rep. Ser. med. Res. Coun., Lond., No. 205.

Consden, R. and Stanier, W.M. (1952) Biochem. J. 51, XIX.

Di Carlo, F. J. and Redfern, S. (1947) Arch. Biochem., 15, 333.

Di Carlo, F. J. and Redfern, S. (1948) Arch. Biochem., 17, 1.

Dische, Z. and Shattles, L.B. (1948) J. biol. Chem., 175, 595.

Doetsch, R.N., Robinson, R.Q. and Shaw, J.C. (1952) J. Anim. Sci., 11, 536

Duguid, J.P. (1948) J. Path. Bact., 60, 265.

Duguid, J.P. (1951) J. Path. Bact., 63, 673.

Elsden, S.R. (1945) J. exp. Biol., 22, 51.

Elson, L.A. and Morgan, W.T.J. (1933) Biochem. J., 27, 1824

Fuller, A.T. (1938) Brit. J. exp. Path., 19, 130.

Gall, L.S., Stark, C.N., Looslie, J.K. (1947) J. Dairy Sci., 30, 891.

Gall, L.S., Burroughs, E.W., Gerlaugh, P. and Edgington B.H. (1949) J. Anim. Sci., 8, 441.

Gall, L.S. and Huhtanen, C.N. (1951) J. Dairy Sci., 4, 353.

Garton, G.A. (1951) J. exp. Biol., 28, 358

Gray, F.V. (1947) J. exp. Biol., 24, 15.

Griffiths, F. (1934) J. Hyg., Camb., 34, 542.

Gruby and Delaford. (1843) Rec. Med. Vet., 20, 859.

Hale, E.B., Duncan, C.W., and Huffman, C.F. (1947) J. Nutrit., 34, 733.

Hanson, S.W.F., Mills, G.T. and Williams, R.T. (1944) Biochem. J., 38, 274.

Hastings, E.G. (1944) Bact. Rev., 8, 235.

Heald, P.J. (1951) Brit. J. Nutrit., 5, 84.

Heidelberger, M. and Kendall, F.E. (1936) J. Immunol. 30, 267.

Heidelberger, M., Kendall, F.E. and Sherp, H.W. (1936) J. exp. Med., 64, 559.

Henneberg, W. (1922) Zbl. Bakt. (2. Abt.) 55, 242.

Hobson, P.N., Whelan, W.J. and Peat, S. (1950) J. chem. Soc., p. 3566.

Hobson, P.N., Pirt, S.J., Whelan, W.J., and Peat, S. (1951) J. chem. Soc., p. 801.

Hockenhull, D.J.D., and Herbert, D. (1945) Biochem. J., 39, 102.

Hofmann, T. (1953) Biochem. J., 54, 293.

Hopkins, R.H. and Kulka, D. (1942) J. Inst. Brew., 48, 107.

Hopkins, R.H., Dolby, D.E. and Stopher, E.G. (1942) J. Inst. Brew., 48, 174.

Hough, L., Jones, J.K.N. and Wadman, W.H. (1952) J. chem. Soc.,p.3393.

Huhtanen, C.N., Rogers, M.R. and Gall, L.S. (1952) J. Bact., 64, 17.

Huhtanen, C.N. and Gall, L.S. (1953) J. Bact., 65, 548.

Huhtanen, C.N. and Gall, L.S. (1953) J. Bact., 65, 554.

Hungate, R.E. (1942) Biol. Bull. Wood's Hole., 83, 303.

Hungate, R.E. (1943) Biol. Bull. Wood's Hole., 84, 157.

Hungate, R.E. (1946) J. Bact., 51, 51.

Hungate, R.E. (1947) J. Bact., 53, 631.

Hungate, R.E. (1950) Bact. Rev., 14, 1.

Ingram, M. and McGaughey, G.A. (1948) Nature, Lond., 162, 534.

Johns, A.T. (1951) J. gen. Microbiol., 5,317.

Johnson, B.C., Hamilton, T.S., Robinson, W.B., and Garey, J.C. (1944) J. Anim. Sci., 3, 287.

Kendall, F.E., Heidelberger, M. and Dawson, M.H. (1937) J. biol. chem., 118, 61.

Kent, P.W. (1952) Chem. Industr. Rev., P. 1176.

Kohler, W. (1940) Archiv. Mikrobiol., 11, 432.

Kon, S.K., and Porter, J.W.G. (1947) Nutr. Abstr. Rev., 17, 31.

Lancefield, R.C. (1933) J. exp. Med., 57, 571.

Loewenthal, H. (1938) Brit. J. Path., 19, 164.

Mangold, E. and Schmidt-Krahmer, C. (1927) Biochem. Z., 191, 411.

Mangold, E. (1929) Handbuch der Ernährung vol.II Julius Springer, Berlin.

Marston, H.R. (1945) Biochem. J., 42, 564.

Masson, M. (1951) Brit. J. Nutrit., 4, viii.

McDonald, I.W. (1952) Biochem. J., 51, 86.

McDougall, E.I. (1948) Biochem. J., 43, 99.

McGaughey, G.A. and Sellars, K.C. (1948) Nature, Lond., 161, 104.

Mackie, T.J. and McCartney, J.E. (1950) Handbook of Practical Bacteriology, Edinburgh, 8th. Ed.

McNaught, M.L., Smith, J.A.B., Henry, K.M. and Kon, S.K. (1950) Biochem. J., 46, 32.

MacPherson, C. (1948) Can. J. Res., 26, 197.

Meyer, K.H., Fuld, M., and Bernfeld, P. (1947) Experientia. 3, 411.

Mills, R.C., Booth, A. N., Bohstedt, G. and Hart, E.B. (1942) J. Dairy Sci., 25, 925

Moir, R.J. and Williams V.J. (1950) Aust. J. Sci. Res., B, 3, 381.

Moir, R.J. (1951) Aust. J. agric. Res., 3, 322.

Moir, R.J. and Masson, M. (1952) J. Path. Bact., 64, 343.

Morgan, W.T.J. (1937) Biochem. J., 31, 2003.

Mowry, H.A. and Becker, E.R. (1930) Iowa State Coll. J. Sci., 5, 35.

Müller, M. (1906) Pflugers Arch. ges. Physiol., 112, 245.

Nelson, N. (1944) J. biol. Chem., 153, 375.

Neufeld, F. (1902) Zft. Hyg. InfektKr., 40, 54.

Oudin, J. (1952) Methods in med. research. Vol. 5. Chicago, The Year Book Publishers.

Oxford, A.E. (1951) J. gen. Microbiol., 5, 83.

Pearson, R.M. and Smith, J.A.B. (1943) Biochem. J., 37, 153.

Phillipson, A.T. and Innes, J.R.M. (1939) Quart. J. exp. Physiol., 29, 333.

Pilgrim, A.F. (1948) Aust. J. Sci. Res.,B, 1, 130.

Pochon, J. (1933) Trav. Stat. Zoo. Wimeraux, 13, 575.

Pochon, J. (1936) Compt. Rend. Soc. Biol., 202, 1538.

Porges, O. (1905) Wein.klin. Wschr., 18, 691.

Pringsheim, H. (1912) Z. Physiol. Chem., 78, 266.

Pounder, W.D. and Hibbs, J.W. (1950) J. Dairy Sci., 33, 639.

Quin, J.I. (1943) Ondestepoort J. vet. Sci., 18, 91.

Ried, J.T. and Huffman, C.F. (1949) J. Dairy Sci., 32, 123.

Rose, D. (1948) Arch. Biochem.,16, 349.

Rowlands, S. (1914) J. Hyg. Camb., 13, Plague Suppl., 3, 418.

Schwarz, C. (1925) Biochem. Z., 156, 130.

Seastone, C.V. (1939) J. exp. Med., 17, 361.

Sevag, M.G. (1934) Biochem. Z., 273, 419.

Sharpe, M.E. and Shattock, P.M.F. (1952) J. gen. Microbiol., 6, 150.

Shattock, P.M.F. and Hirsch, A. (1947) J. Path. Bact.,59, 495.

Shattock, P.M.F. (1949) J. gen. Microbiol., 3, 80.

Sjipesteijn, A.K. (1948) Thesis. Leiden.

Smith, J.A.B. (1945) Proc. Nutr. Soc., 3, 203.

Smith, V.R. (1941) J. Dairy Sci., 24, 659.

Smith, J.A.B. and Baker, F. (1944) Biochem. J., 38, 496.

Starke, E. and Tetrault, P.A. (1951) Canad. J. Bot., 29, 91.

Starke, E. and Tetrault, P.A. (1951) J. Bact., 62, 247.

Somogyi, M. (1945) J. biol. Chem., 160, 61.

Somogyi, M. (1952) J. biol. Chem., 195, 19.

Tappeiner, H. (1882) Ber. dtsch. chem. Ges., 15,999.

Tappeiner, H. (1884) Z. Biol., 20, 52.

Thaysen, A.C. and Bunker, H.J. (1927) Microbiology of cellulose, hemi-cellulose, pectin and gums. Oxford Univ. Press, Lond.

Tóth, L. (1948) Experientia., 4, 395.

Trier, H.J. (1926) Z. vergl. Physiol., 4, 305.

Thaysen, A.C. and Galloway, L.D. (1930) Microbiology of starch and sugars, P. 18.

Usuelli, F. and Fiorni, P. (1938) Boll. soc. ital. Biol. sper., 13, 11.

Washburn, L.E. and Brody, S. (1937) Univ. Missouri. Coll. Agric., Agric. Exp. Stat. Res. Bull. No.263.

Wath, J.G. van der, and Myburgh, S.J. (1941) Ondestepoort J. vet. Sci., 17, 61.

Wath, J.G. van der (1948) Ondestepoort J. vet. Sci., 23, 367.

Watson, R.H. (1933) Aust. J. exp. Biol. med. Sci., 11, 67.

Watson, R.H. and Jarrett, I.G. (1945) Aust. Vet. J., 21, 8.

Westhuizen, G.C.A.van der, Oxford, A.E., Quin, J.I. (1950) Ondestepoort J. vet. Sci., 24, 119.

Whelan, W.J. and Nasr, H. (1951) Biochem. J., 48, 416.

Woodman, H.E. and Stewart, J. (1928) J. agric. Sci., 22, 527.

Woodman, H.E. and Stewart, J. (1932) J. agric. Sci., 22, 527.

Zunts, N. (1891) Pflug. Arch. ges. Physiol., 49, 483.

ACKNOWLEDGEMENTS

The author wishes to express her thanks to Dr. A. E. Oxford for his helpful guidance and criticism.

Thanks are also due to the following members of staff of the Rowett Research Institute, Drs. A. T. Phillipson, D. Duncan, and A. Iggo for providing fistulated sheep, to Miss M. Masson for assistance with photo-micrography and to Mr. S. Mann for assistance in the preparation of immune sera.

(*Reprinted from Nature, Vol.* 171, *p.* 129, *January* 17, 1953)

Encapsulation in Rumen Bacterial Fractions

METHODS of fractionating the rumen contents of sheep previously described in the literature (see, for example, footnote 1) involve centrifugation of uncooled, strained, rumen liquids at varying speeds on centrifuges running at room temperature. These procedures were found by us to be unsatisfactory for two reasons. First, during the time between collection and centrifugation of the liquids, the composition of the sample might alter as a result of bacterial fermentation and autolysis ; and secondly, during the centrifugation, especially at high speeds, the liquids warm up to as much as 40° C., thus setting up convection currents in the centrifugate and causing fermentation with the liberation of gases which break up the sediments. We have found that these difficulties are overcome by the following procedure, which allows of fractionation on a small scale.

Rumen contents were collected through a permanent rumen fistula[2] by means of a wide-bore glass tube fitted with a rubber suction bulb, and immediately pipetted into a conical flask cooled in a freezing mixture of ice and salt, which reduced the temperature of the rumen contents to *c.* + 1° C. in 10–15 min. Further operations were then carried out in a cold room at + 1° C. The rumen contents were strained through a 60-gauge copper gauze to remove plant particles. (It is well to note that, if the fractions are required for cultural studies, the copper gauze should be replaced by bolting silk, as small traces of copper have a bactericidal effect.) The resulting liquid was centrifuged for 20 min. at 22,500 *g* in a 'Superspeed' head on a refrigerated centrifuge (M. S. E., London), conditions being adjusted so that the rumen liquid remained at + 1° to + 3° during centrifugation. If the temperature was further lowered, ice began to separate from the liquid.

Two sheep were used in the experiments. One was fed mixed concentrates (250 gm.) and potato starch (100 gm.), twice daily, with one feed of hay (250 gm.) in between, and the other was fed on hay (*ad lib.*). The rumen contents were sampled three hours after the morning feed. The samples from the starch-fed sheep were thick and formed four distinct fractions on centrifuging. These are described in the accompanying table.

Fractions 1 and 2 from the rumen liquids of the hay-fed sheep were similar to those from the starch-fed animal, but fractions 3 and 4 formed a single

Fraction No.	Appearance	Constituents
1	Brown opalescent liquid	Almost bacteria-free; few Gram-positive cocci and Gram-negative bacilli
2	White semi-solid layer	Almost entirely Gram-negative bacilli, cocci and vibrios; few Gram-positive cocci and bacilli
3	Fine green compact sediment	Protozoa, selenomonas and bacteria, as in fraction 2, plant particles and starch granules
4	Coarse material	Mostly plant particles, with some bacteria and protozoa

layer. The sediments were easily separated by repeated washing with a small volume of phosphate buffer (0·1 *M*; *p*H 7·0). A photomicrograph of a typical bacterial fraction (2) from the rumen liquids of a starch-fed sheep is shown in Fig. 1.

Examination of fraction 2 by Duguid's indian ink technique[3] showed that large numbers of the bacteria were heavily encapsulated, a fact which has not been noted in fractions prepared by the usual methods. The largest capsules, up to 9 μ in transverse diameter, were found on the bacteria from the hay-fed sheep. In the fraction from the starch-fed sheep large clumps of agglutinated bacteria surrounded by capsular material were seen. Moir and Masson[4] have recently described a survey of whole rumen contents, either fresh or incubated *in vitro* for one hour with glucose, in which they noted some encapsulated organisms. The present work suggests that encapsulated organisms constitute a large part of the bacterial fractions.

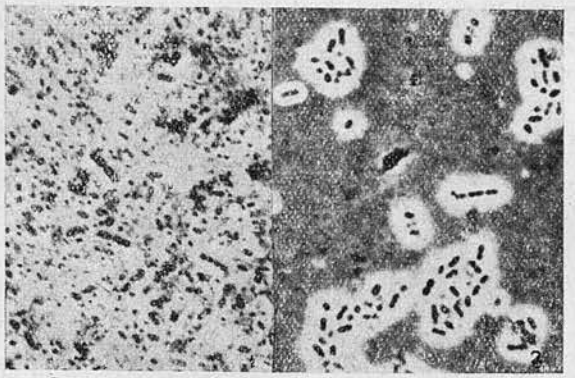

Fig. 1. Bacterial fraction 2 from a sheep on a starch-containing diet. Stained by Gram's method. × 1,500

Fig. 2. Bacterial fraction 2 from a sheep on a starch-containing diet. Wet indian ink preparation, more dilute than Fig. 1, showing extensive encapsulation. Phase contrast. × 1,650

This capsular material appears, from preliminary experiments on a strain of rumen streptococcus isolated in pure culture[5], to contain polysaccharide which, like the majority of the bacterial capsular materials so far investigated, does not stain with iodine. However, whether this capsular material can be utilized by the host animal at a later stage in the digestion cycle, as can starch-type intracellular polysaccharides[6], or can be converted by other bacteria into products of value to the host cannot yet be stated.

We wish to thank Dr. A. E. Oxford for interest shown in this work and for placing the hay-fed sheep at our disposal.

P. N. Hobson
Margaret J. MacPherson

Rowett Research Institute,
Bucksburn,
Aberdeenshire.
July 21.

[1] Smith, J. A., and Baker, F., *Biochem. J.*, **38**, 496 (1944). Johnson, B. C., Hamilton, T. S., Robinson, W. B., and Garey, J. C., *J. Animal Sci.*, **3**, 237 (1944). Heald, P. J., *Brit. J. Nutrit.*, **5**, 84 (1951).

[2] Phillipson, A. T., and Innes, J. R. M., *Quart. J. Exp. Physiol.*, **29**, 333 (1939).

[3] Duguid, J. P., *J. Path. Bact.*, **60**, 265 (1948).

[4] Moir, R. J., and Masson, M. J., *J. Path. Bact.*, **64**, 343 (1952).

[5] MacPherson, Margaret J., and Oxford, A. E., *J. Gen. Microbiol.*, **1**, ii (1952).

[6] Baker, F., *Nature*, **150**, 479 (1942).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

[From THE BIOCHEMICAL JOURNAL, Vol. 52, No. 4, pp. 671–679, 1952]
 PRINTED IN GREAT BRITAIN

Amylases of *Clostridium butyricum* and a *Streptococcus* Isolated from the Rumen of the Sheep

By P. N. HOBSON and MARGARET MACPHERSON
The Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 13 *March* 1952)

In the course of an investigation into the digestion of starch by the pig, Baker, Nasr, Morrice & Bruce (1951) isolated a micro-organism responsible for the breakdown of starch in the caecum and identified it as a strain of *Clostridium butyricum*. Later Masson (1951) isolated another strain of *Cl. butyricum* which was the chief agent of starch breakdown in the rumen of a sheep fed on a flaked maize and hay diet. Continuing these investigations into the digestion of starches by the sheep, MacPherson (forthcoming publication) obtained several strains of a *Streptococcus*, probably related to *Strep. bovis*, from the rumen, one being selected for use throughout this work. The extracellular amylase formed when the pig caecum strain of *Cl. butyricum* was grown on a medium containing soluble starch was examined by Whelan & Nasr (1951) and was shown to be of the α-amylase type. Both the sheep rumen *Clostridium* and *Streptococcus* formed an extracellular amylase when grown in a medium containing dissolved starch, and an investigation of these amylases is the subject of this paper. The amylases would appear to be constitutive and not adaptive enzymes since, when the *Clostridium* was grown in a medium containing maltose as the only carbohydrate, and the *Streptococcus* on a glucose-containing medium, the other constituents being the same as in the starch medium, amylase activity was present in the cell-free filtrates.

There are at present known to be two major groups of starch-hydrolysing enzymes, the α- and β-amylases. In addition, there is the amylase of *Bacillus macerans* which has a synthetic as well as a hydrolytic function, as it catalyses the synthesis of cyclic 'Schardinger dextrins' from starch and starch fractions. Recently, also, various authors have reported the isolation of a maltase from a bacterium and moulds which is also capable of hydrolysing starch entirely to glucose (French & Knapp, 1950; Kerr, Cleveland & Katzbeck, 1951; Philips & Caldwell, 1951*a*, *b*). The only sugar produced during the hydrolysis of starch-type polysaccharides by β-amylase, which is found in ungerminated cereals, is maltose. Until recently it was thought that amylose, the 'linear' component of starch, was entirely

converted to maltose by this enzyme, but recent work by Peat, Pirt & Whelan (1952) has shown that the purest samples of β-amylase produce only 70% of the theoretical maltose from amylose. The presence of a second enzyme (Z-enzyme), usually present in β-amylase preparations, and which has been shown to be a β-glucosidase (Peat, Thomas & Whelan, 1952), is needed to enable the hydrolytic action of the β-amylase to continue beyond this arrest point. β-Amylase degrades the polysaccharide chain from the non-reducing end group and its action is arrested by the presence of the α-1:6-linkages in amylopectin, the 'branched-chain' component of starch, leading to the formation of a limit dextrin, 'β-dextrin'. The α-amylases on the other hand degrade starch and starch fractions in a more random manner and can hydrolyse the α-1:4-linkages of the inner chains of amylopectin between the α-1:6-branch linkages forming first dextrins and then a mixture of reducing sugars. This property of the α-amylases is generally used for testing for their presence in β-amylase preparations. Pure β-amylase does not attack β-dextrin, but a small concentration of α-amylase will hydrolyse α-1:4-linkages beyond the branch points, thus exposing more non-reducing end groups to the action of the β-amylase and resulting in degradation of the dextrin. The presence of β-amylase in α-amylase preparations is more difficult to detect, but Hobson, Whelan & Peat (1950) have recently introduced a method for characterizing α-amylase by comparing its actions on amylose and β-dextrin and this has been applied to the amylases described in this paper. Quantitative determinations of amylase activity depend on the measurement of either the reducing sugars formed, or the change in iodine stain of starch fractions under standard conditions. It is usual to measure the maltose formed by β-amylase, while, since the reducing power of the initial products of α-amylolysis is small, α-amylase activity is generally determined by observing the time taken for a starch digest to attain a standard iodine colour, or by measuring the change in iodine colour of the digest over a standard time. It should be emphasized, however, that the presence of one amylase as impurity can lead to erroneous results in measuring the activity of the other.

The bacterial amylases so far studied have, with the exception of the *B. macerans* enzyme, been of the α-type. The amylase of *B. subtilis* was investigated by Hopkins & Kulka (1942), and Hopkins, Dolby & Stopher (1942), and by Di Carlo & Redfern (1947*a*, *b*, 1948). This enzyme has now been obtained in a crystalline form by Meyer, Fuld & Bernfeld (1947) and in its saccharifying and dextrinizing action on starch it appears to be similar to salivary, pancreatic and malt α-amylases, but the nature of the end products of the hydrolysis have not been reported. Hopkins *et al.* (1942), however, showed that the *B. subtilis* enzyme was an α-amylase and that it was similar to malt α-amylase in that it rapidly hydrolysed starch until the sugars liberated corresponded to 40% apparent maltose. Thereafter the rate of hydrolysis was slower until a limiting conversion of 75–80% apparent maltose was found. Rose (1948), investigating the properties of an amylase formed by *B. polymyxa*, found that the reducing power of the products of the hydrolysis of starch by this enzyme did not exceed 80% theoretical maltose. Hockenhull & Herbert (1945) were not able to obtain a maltase-free enzyme from *Cl. acetobutylicum* and, although the rate of hydrolysis decreased when about 70% apparent maltose had been liberated, conversions of starch to considerably greater values than 100% apparent maltose were observed. On the basis of 100% conversion of starch to glucose by the maltase-containing enzyme it was concluded that the α-amylase hydrolysed starch completely to maltose. However, the recent work of French & Knapp (1950) renders this conclusion invalid as they have shown that the maltase of *Cl. acetobutylicum* alone converts starch and dextrins entirely to glucose. More recently, Whelan & Nasr (1951) described the properties of the amylase of a strain of *Cl. butyricum* isolated from the pig caecum. The enzyme was an α-amylase which rapidly degraded potato starch and amylose until an apparent conversion to maltose of 65–66% was reached. Thereafter the hydrolysis ceased.

METHODS AND MATERIALS

Determination of reducing sugars. The copper reagent of Somogyi (1945*a*) standardized against maltose solution was used. Before being added to the reagent portions of the digests containing the *Cl. butyricum* amylase preparations were neutralized with a predetermined volume of 1 N-NaOH and deproteinized with $ZnSO_4$ and $Ba(OH)_2$ as described by Somogyi (1945*b*) as the enzyme preparation was, otherwise, found to lower the apparent maltose concentration. Since the streptococcal amylase preparations had only a slight effect on the reagent it was found more convenient to standardize the Somogyi reagent against maltose solutions containing the enzyme preparation in the concentrations of the digests and to determine the reducing power of the digest samples from these standard graphs, after neutralization with a predetermined volume of 1 N-NaOH. The iodometric technique was used throughout.

Measurement of the iodine stain of polysaccharides. (1) *Blue value* (*BV*). The blue value of a polysaccharide is defined as the reading on the logarithmic scale of a Spekker photoelectric absorptiometer (Hilger and Watts, London), when the absorption of light (relative to water) by a solution of a polysaccharide-iodine complex contained in a 4 cm. cell and having the composition, polysaccharide (1 mg.), I_2 (2 mg.), KI (20 mg.)/100 ml. is measured using Ilford gelatin filter 608 transmitting light of wavelength 680 mμ. (see Bourne, Haworth, Macey & Peat, 1948). The blue value is thus a characteristic of the starch fraction. (2) *EEL blue value* (*EBV*). In this work the light absorption of the polysaccharide-iodine solutions of the concentrations given above contained in a standard $\frac{5}{8}$ in. diameter colorimeter tube was measured in an 'EEL' colorimeter (Evans Electroselenium Ltd., Harlow, Essex) using filter 205 transmitting light of wavelength 680 mμ. A reading of 51·2 on the EEL scale is equivalent to a Spekker reading of 1·40. Thus 1 *EBV* is equivalent to 36·6 *BV*. (3) *Absorption value.* (*AV*). The absorption value is a measurement of the iodine stain of a polysaccharide solution made in the same way as a blue value except that the wavelength of the light used and the polysaccharide concentration are not standard. The wavelength of light used is stated whenever an absorption value is quoted, but the concentration of polysaccharide may be unknown, as, for example, during an amylolysis. (4) '*EEL' value* (*EV*). This is an absorption value measured on the EEL colorimeter instead of a Spekker absorptiometer.

Fractionation of potato starch. Amylose was obtained from potato starch by the $Al(OH)_3$-thymol method of Hobson, Pirt, Whelan & Peat (1951). Amylopectin was prepared by fractionation of thymol-amylopectin with methanol as described by these authors. All starch fractions were dried *in vacuo* at 60° over P_2O_5 before use.

Preparation of β-dextrin. This was prepared by the hydrolysis of thymol-amylopectin with soya-bean β-amylase as described by Hobson, Whelan & Peat (1950).

Measurement of α-amylase activity. A colorimetric method was used for determination of the amylase activity, as this was found to need less enzyme than the method previously described where the reducing power of an amylase-starch digest was measured after incubation under standard conditions (Hobson, quoted by Whelan & Nasr, 1951). β-Dextrin was used in preliminary experiments, but it was found that the fall in *EV* (680 mμ.) was proportional to the amount of enzyme present only over a small range, so amylose, of blue value 1·2, was substituted for the dextrin. The amylose (approx. 50 mg.) was dissolved in 0·1 N-NaOH (10 ml.) by heating in a boiling-water bath for not more than 9 min. After cooling, the solution was made just acid to phenolphthalein with 5 N-H_2SO_4 and appropriately diluted. The volume added to the digest mixture was arranged to bring the final concentration of amylose in the digest to 1 mg./ml. The standard digest mixture contained 3·75 ml. amylose, 1·5 ml. of 0·2 M-sodium citrate buffer, pH 7·0, and enzyme solution (1·75 ml.), the digest contents being preheated to 35° before mixing. The digest was incubated at 35° for 30 min. when 1 ml. was removed and stained with I_2 (2 mg.), KI (20 mg.)/100 ml. water for measurement of the *EV* (680 mμ.). This *EV* (680 mμ.) was compared with that of a similar portion of a blank digest containing water in place of the enzyme, and, providing the fall in *EV* (680 mμ.) was not more than 24 units on the colorimeter scale it was strictly proportional to the amount of enzyme present. One activity unit is defined as the amount of amylase which will produce a fall in *EV* (680 mμ.) of one colorimeter scale unit under the above conditions.

Standard digests for determination of the action of the enzyme preparations on polysaccharides. Solutions of starch or starch fractions were prepared as above and incorporated in digests containing 7·5 ml. polysaccharide solution (14 mg.), 0·2 M-sodium acetate buffer of the appropriate pH value (3·0 ml.), water (1·5 ml.), and enzyme solution (2 ml.; activity 66 units). The digest was incubated at 35° and at intervals portions (usually 3 or 5 ml.) were removed for determination of the reducing power, against portions of a blank digest, containing water in place of the polysaccharide solution, which was incubated in parallel. Where necessary the digest volumes were increased to enable more determinations to be made.

Human salivary α-amylase. Saliva was collected as needed, diluted with an equal volume of water and centrifuged to remove the mucin. A portion of the supernatant liquid was then diluted to give a solution of the required activity.

Preparation of cell-free filtrates. (1) *Cl. butyricum.* Cultures of the *Clostridium* were inoculated into a modified Beijerinck fluid medium (400 ml.) containing 5% soluble starch (Baker *et al.* 1951) and incubated at 40° for 40 hr. After this time all iodine-staining polysaccharide in the medium had been degraded and gas production had almost ceased. The culture was centrifuged for 30 min. at 740 *g* to remove most of the cells, and then passed through a Seitz filter fitted with a Carlson 'EK' sterilizing pad. The volume of sterile filtrate obtained was usually about 300 ml. (2) *Streptococcus.* The medium used for these organisms was a yeast broth (200 ml.; 1% 'Difco' yeast extract, 1% Bacto Tryptone, 0·1% KH_2PO_4, pH adjusted to 6·8) containing 5% dissolved starch and 3% (w/v) $CaCO_3$. The culture was incubated at 40° for 40 hr. with occasional shaking to suspend the $CaCO_3$. At this time amylase activity was maximal. The culture was then centrifuged until clear (20 min. at 1300 *g*) and sterilized by filtration as above. The volume of filtrate obtained was about 150 ml.

Preparation of freeze-dried enzymes. Preliminary experiments were carried out to determine the optimum concentrations of $(NH_4)_2SO_4$ for precipitation of the enzymes. All precipitations were carried out as near as possible to +1°. Freeze drying was carried out in 1 l. round-bottomed flasks at 0·1 mm. pressure using an acetone and solid carbon dioxide condenser. (1) *Cl. butyricum.* In a typical preparation an initial precipitation of the enzyme from 275 ml. of culture filtrate was carried out by addition of $(NH_4)_2SO_4$ solution (50% w/v; pH adjusted to 7·0 with NH_4OH) to 40% (w/v) and the precipitate freeze-dried. This precipitate (2·49 g.) was then dissolved in water (100 ml.; solution 1) and $(NH_4)_2SO_4$ solution added slowly to a concentration of 30% (w/v). After storing for 1·5 hr. the precipitate was compacted on the centrifuge in cold pots, washed with cold 30% (w/v) $(NH_4)_2SO_4$ solution, dissolved in 0·02 M-sodium citrate buffer (25 ml.; pH 7·0), and freeze-dried to give fraction *A*. Some $(NH_4)_2SO_4$ was added to the supernatant liquid to bring the concentration to 40% (w/v). The precipitate was removed after 4·5 hr., washed, dissolved in citrate buffer, as above, and freeze-dried to give fraction *B*. The initial activity of the solution (1) was 19 800 units, the activities of fractions *A* and *B* before freeze-drying were 17 000 and 1300 units respectively. After freeze-drying the weight of fraction *A* was 0·4609 g. and the activity 13 980 units. The weight of freeze-dried fraction *B* was 1·6296 g., but the activity was not determined. (2) *Streptococcus.* In a typical large-scale preparation $(NH_4)_2SO_4$ was slowly added to the filtrate (152 ml.) to a concentration of 40% (w/v). After standing at +1° for 14·5 hr. the fine precipitate was removed on the centrifuge, washed and dissolved in water (100 ml.). More $(NH_4)_2SO_4$ was then added to a concentration of 35% (w/v) and after storing at +1° for 4·5 hr a fine suspension formed which was centrifuged off, washed with 35% $(NH_4)_2SO_4$, dissolved in 0·02 M-sodium citrate buffer (25 ml.; pH 7·0) and freeze-dried (fraction *A*). The weight of freeze-dried powder obtained was 0·6923 g. and the total activity 13 140 units compared with 13 200 units before freeze-drying. This was about half the activity of the original filtrate.

Fraction *A* from each filtrate was used in the following work. These powders kept their activity indefinitely when stored at +1°.

RESULTS

The action of the enzyme preparations on amylose and β-dextrin

Hobson *et al.* (1950) showed that by incubating equal amounts of α-amylase with (1) amylose and (2) β-dextrin and plotting the percentage change in *AV* (680 mμ.) of the amylose-iodine complex against that of the β-dextrin, a curve characteristic of the enzyme and independent of its concentration could be obtained. Two standard digests, both containing the same enzyme preparation, but one containing amylose and the other β-dextrin, were

incubated together, and, at equal periods after addition of the enzyme, portions were removed for determination of the *EV* (680 mμ.). The sample from the amylose digest was stained under the standard conditions given above, but that from the β-dextrin digest contained twice the normal concentrations of polysaccharide and was stained with twice the normal concentration of iodine to increase the accuracy of the measurements.

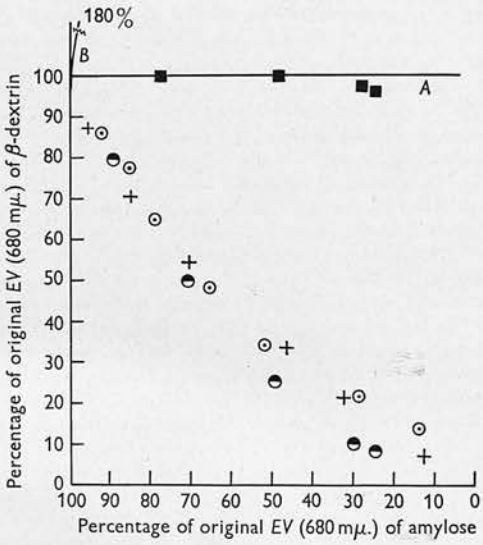

Fig. 1. The comparative action of the enzymes on amylose and β-dextrin. *A*, theoretical curve for β-amylase and Q-enzyme. *B*, curve for R-enzyme. Soya-bean β-amylase (■), clostridial amylase (+), salivary amylase (◓), at pH 7·0. Streptococcal amylase (⊙), at pH 6·0. All digests incubated at 35°.

Q-enzyme and β-amylase attack amylose but not β-dextrin so that a single straight line serves to represent the action of these enzymes. R-enzyme, which catalyses the hydrolysis of the α-1:6-linkages in β-dextrin and amylopectin, acts on β-dextrin with the formation of dextrins of higher blue value (Hobson, Whelan & Peat, 1951), and its action is represented by the curve shown in Fig. 1. α-Amylase such as salivary amylase is the only enzyme which attacks both substrates and the curve depicting its action is shown in Fig. 1 where the results of experiments using salivary α-amylase and the clostridial and streptococcal enzymes are plotted. Mixtures of α-amylase with other enzymes give curves of intermediate shape.

Optimum pH *and temperature of action of the enzymes.* The optimum pH of action was measured by determining the fall in *EV* (680 mμ.) of digests like the standard-activity digests but containing a sodium acetate-sodium veronal buffer the pH of which had been adjusted by the addition of 0·1 N-H_2SO_4 (5 ml. 0·143 M-sodium acetate-sodium veronal solution, *x* ml. acid, (18 − *x*) ml. water). The constituents were preheated to 35°, mixed, and the *EV* (680 mμ.) of portions of the digests were measured after incubation for 15 and 30 min. as in the determination of activity. The pH values of the digests were checked at the end of the incubation.

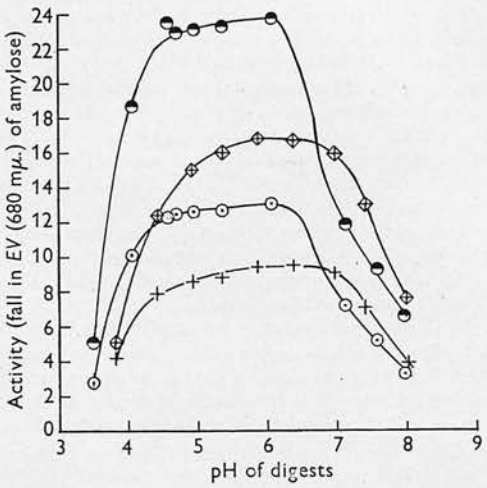

Fig. 2. The effect of pH on amylase activity at 35°. Incubation periods: clostridial amylase, 15 min. (⊙), 30 min. (◓); streptococcal amylase, 15 min. (+), 30 min. (⊕).

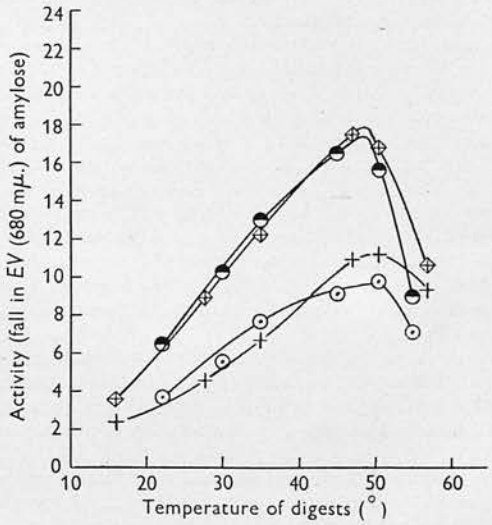

Fig. 3. The effect of temperature on amylase activity. Digests at pH 7·0. Incubation periods: clostridial amylase, 15 min. (⊙), 30 min. (◓); streptococcal amylase, 15 min. (+), 30 min. (⊕).

Both enzymes have a broad pH optimum. That of the streptococcal enzyme under these conditions lies between 5·5 and 6·5 and that of the clostridial enzyme between 4·6 and 6·1 (Fig. 2). This latter value is similar to the optimum pH range of

crystalline *B. subtilis* enzyme (5·3–6·8; Meyer *et al.* 1947), partially purified *B. subtilis* amylase (5–6·5; Di Carlo & Redfern, 1947*a*) and *B. polymyxa* amylase (6·2–7·5; Rose, 1948).

The optimum temperature was determined by incubating the standard activity digests at different temperatures and determining the fall in *EV* (680 mμ.) after 15 and 30 min. in the usual manner.

The results in Fig. 3 show that both enzymes have the same optimum temperature of 48 ± 1° under the digest conditions employed. For subsequent experiments a temperature of action of 35° was chosen for both enzymes.

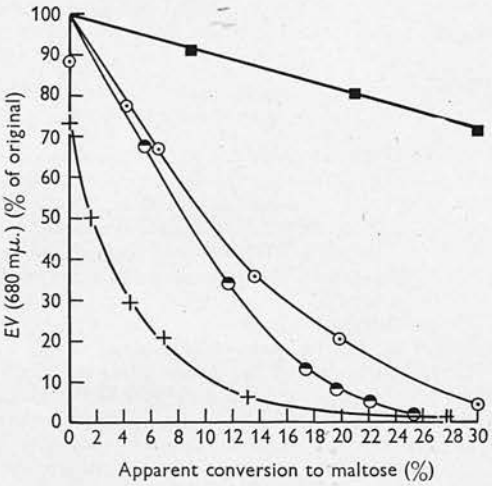

Fig. 4. The relationship between *EV* (680 mμ.) and percentage apparent conversion to maltose at 35° for amylose degraded by the clostridial amylase at digest pH values of 4·8 and 7·0 (+); streptococcal amylase at pH 6·0 (⊙); salivary amylase at pH 7·0 (◑). Typical β-amylase curve (■).

The action of the enzymes on amylose

The relationship between absorption value and percentage apparent conversion to maltose. An amylose of blue value 1·21 was used in these experiments to facilitate comparison of the results with those obtained by Whelan & Nasr (1951), as it was found that slightly different curves were obtained when amylose fractions of different blue values were used. Digests of the standard proportions, but with the total volume increased to 42 ml., were employed. A buffer of pH 7·0 was used for salivary α-amylase and two, of pH 7·0 and 4·8, for the clostridial amylase, the same curve being obtained at both pH values. For the streptococcal enzyme a buffer of pH 6·0 was used. Withdrawals for measurement of *EV* (680 mμ.) (see Methods section), and for measurement of reducing power, as maltose, were made alternately at noted intervals and from the progress curve of fall in *EV* with time the values corresponding to the respective reducing powers were found.

The results are shown in Fig. 4, a typical curve obtained with β-amylase being included for comparison.

Tests for the presence in the preparations of enzymes other than α-amylase

β-Amylase, Q- and R-enzymes. The results obtained from the comparison of the action of the enzyme preparations on amylose and β-dextrin obviate the presence of more than traces of β-amylase, Q- and R-enzymes.

Maltase. The polysaccharide solution in the standard digests was replaced by a maltose solution of the same concentration. The action of the clostridial enzyme on maltose was tested at pH values of 4·8 and 7·0 and that of the streptococcal enzyme at pH 6·0. Portions of the digests were removed for determination of the reducing powers, as maltose, at approximately 10 hr. intervals up to 48 hr.

No change in reducing power was found and the enzyme preparations were therefore free from maltase. Quantitative determinations, using portions of the digests as enzyme source, showed that the enzymes in the digests containing maltose retained their initial activity after incubation for 48 hr.

Phosphorylase. Phosphorylase activity was determined using the method of Green & Stumpf (1942) except that since the phosphorylase activity of the enzyme preparations was small, incubation was carried on for 1 hr. before the deactivation of the digests by addition of trichloroacetic acid. For determination of the initial concentration of inorganic phosphate a digest containing all the constituents but the enzyme was incubated in parallel, then trichloroacetic acid was added, and, immediately after, the enzyme preparation. Both digests were centrifuged and the inorganic phosphate in a suitable sample determined by the method of Allen (1940).

Experiments using the enzyme fractions obtained at 30 and 40% (w/v) concentrations of ammonium sulphate in the preliminary fractionation of the clostridial amylase showed that the fraction precipitated by 30% w/v $(NH_4)_2SO_4$ had a very slight phosphorylase activity, but that the fraction precipitated by 40% w/v $(NH_4)_2SO_4$ had three times as much. For the determination of the phosphorylase activity of the freeze-dried preparations the enzyme was used in a concentration of 220 α-amylase units/digest. The phosphorylase activity of the streptococcal amylase preparation was of the order of 1 unit/13 000 α-amylase units, and of the clostridial amylase 1 unit/10 000 α-amylase units.

Phosphatase. The composition of digests and the experimental conditions were the same as for the determination of phosphorylase activity except that 0·1M-sodium α-glycerophosphate replaced 0·1M-glucose-1-phosphate. Neither enzyme preparation liberated inorganic phosphate from the glycerophosphate. α-Glycerophosphatase activity is therefore absent under these conditions.

Invertase. The polysaccharide in the standard digests was replaced by a sucrose solution of the

same concentration. The buffers used were of pH values 4·8 and 7·0 for the clostridial amylase and 6·0 for the streptococcal enzyme. After incubation for 20 hr. none of the digests showed any reducing power thereby indicating the absence of invertase.

The effect of chloride and calcium ions on the activity of the enzymes

Chloride ions. Two digests were prepared as for the activity determinations except that one contained sufficient NaCl to bring the final concentration to 0·14 M. The digests were incubated at 35° for 30 min. when portions (1 ml.) were removed for determination of *EV* (680 mμ.) as in the determination of activity.

Calcium ions. Two digests were prepared each containing amylose solution (7 mg.; 3·75 ml.), 0·2 M-sodium acetate solution the pH of which was adjusted to 7·0 (1·5 ml.) and enzyme solution (0·75 ml.). In addition, one digest contained $CaCl_2$ solution (1 ml.) to give a final concentration of 0·07 M, and the other NaCl solution (1 ml.) such that the final concentration was 0·14 M. The digests were incubated at 35° and at 30 min. the *EV* (680 mμ.) of portions (1 ml.) of each were measured.

The results are shown in Table 1. The presence of calcium ions causes the slightly higher *EV* of the samples from digests containing calcium chloride. A similar effect is found with starch-iodine solutions containing calcium nitrate. Neither enzyme is activated by calcium or chloride ions under the conditions employed, but in some (unreported) experiments it was found that dilute solutions of the enzymes were stabilized by the presence of small concentrations of calcium ions.

The hydrolysis of starch and starch fractions by the enzymes

Solutions of the amylose, and amylopectin from potato starch were prepared and incorporated in digests of the standard proportions containing buffers of pH values 4·8 and 6·0 for the clostridial amylase and 6·0 for the streptococcal amylase. The digests were incubated at 35°, and, at intervals, the reducing sugars present were determined and expressed as maltose. The activities of the enzymes in the digests were determined at intervals.

After 27 hr. the streptococcal enzyme retained its initial activity in the digests (4·7 units/1 ml.), but at 72 hr. the activity had fallen to 2·9 units/1 ml., and at 118 hr. it was 0·8 unit/1 ml. The clostridial amylase retained its full activity at 24 hr., and the activity at 118 hr. was 1 unit/1 ml. At 72 hr. fresh enzyme was added to portions of each digest containing amylose and, to the digests containing the clostridial amylase, starch and amylopectin to bring the activity to the initial value; the extents of conversion to reducing sugars did not, however, markedly increase above those of the original digests. The apparent conversions to maltose of the different substrates by the two enzyme preparations are shown in Figs. 5 and 6; in each case the values found for starch are almost exactly those calculated from the extent of the hydrolysis of the two constituents, amylose and amylopectin.

Identification of the main products from the hydrolysis of amylose by the streptococcal-enzyme preparation

A standard digest containing 28 mg. of amylose was incubated at 35°, the reducing power being determined at 21 and 23·5 hr. (90·7 % apparent maltose). At 27 hr. 20 ml. of the digest were shaken alternately with cation- and anion-exchange resins (Amberlite 1 R 100/8 and 1 R 4 B; Rohm and Hass Co., Philadelphia, U.S.A.) to remove the buffer and the solution evaporated to dryness under reduced pressure at 45°. The resulting residue was dissolved in water (0·5 ml.) and spots of this solution, to the total volumes given below, were layered on to the starting line of a strip of Whatman no. 1 filter paper. The first chromatogram had spots consisting of approximately 0·01 ml. of amylolysis products, and 0·01 ml. of amylolysis products mixed with 0·005 ml. of 1 % glucose solution, together with reference spots of 0·002 ml. of 1 % glucose, 0·002 ml. of 1 % maltose and 0·002 ml. of 1 % maltotriose. The chromatogram was developed with a solvent consisting of A.R. amyl alcohol (British Drug Houses Ltd.), pyridine and water in the proportions by volume of 7 : 7 : 6 (Jeanes, Wise & Dimler, 1951) for 20·75 hr. at 20°; the solvent, which had run 32 cm., was then dried off, and the chromatogram replaced and developed for a further 19·5 hr., the solvent running the same distance. After drying, the paper was coloured by spraying with a benzidine reagent (Bacon & Edelman, 1951).

Three spots corresponding to glucose, maltose and maltotriose were found in the α-amylolysis products. A similar chromatogram was developed with the top layer of a benzene, *n*-butanol, pyridine, water mixture (in the proportions by volume 10:50:30:30)

Table 1. *The effect of calcium and chloride ions on the activity of the enzymes*

(Digests 1 contained amylose (7 mg.), 0·2 M-sodium citrate (1·5 ml., pH 7·0), and enzyme in a total volume of 7 ml. In digests 2 the sodium citrate was replaced by sodium acetate, and the enzyme activity differed from digests 1. The *EV* gives a measure of the enzyme activity; see text. Initial *EV* of amylose, 42·0.)

	EV (680 mμ.) of amylose after incubation for 30 min. at 35°			
	1		2	
Salts added to digests	0·14 M-NaCl	No salts	0·14 M-NaCl	0·07 M-$CaCl_2$
Source of the enzyme				
A rumen *Streptococcus*	35·0	35·0	32·6	34·1
Clostridium butyricum	34·5	34·5	33·8	35·2

for 44·5 hr. at 20°, the solvent being allowed to drip off the bottom of the paper, and after drying was coloured with the benzidine reagent. Again spots corresponding to glucose, maltose and maltotriose were found in the α-amylolysis products, the separation of the sugars being greater in this solvent. No material of chain length greater than maltotriose was detectable on these chromatograms. The most abundant products of the hydrolysis are thus glucose, maltose and maltotriose, the intensity of the spots suggesting that the maltotriose was present in large amount, and the glucose in comparatively small amount.

The action of the enzyme preparations on maltotriose and maltotetraose

The maltotriose and maltotetraose used were isolated from the end products of hydrolysis of amylose by the clostridial amylase by chromatography on charcoal in the manner of Whistler & Durso (1950) and Bailey, Whelan & Peat (1950). The maltotriose had $[\alpha]_D + 168°$ (in water) and an apparent chain length (from the copper-reducing power measured as maltose) of 2·82 glucose units and gave the theoretical amount of glucose on acid hydrolysis. The maltotetraose had $[\alpha]_D + 176°$ and an apparent chain length of 4·2 and was hydrolysed to the theoretical amount of glucose. No carbohydrate impurities could be detected by chromatography and a graph of $\log (1/R_F - 1)$ per chain length of these sugars with glucose and maltose gave a straight line. The digests were of the same proportions as the standard digests, but containing a known amount (approx. 14 mg.) of the sugar. A buffer of pH 4·8 was used for the clostridial enzyme and of pH 6·0 for the streptococcal enzyme. The reducing power of digests containing maltotriose was measured up to 22 hr. in the case of the clostridial enzyme, and a further reading was taken at 51 hr. in the case of the streptococcal amylase. No increase in reducing power occurred. The results for the digests containing maltotetraose are shown in Table 2. The maltotetraose is slowly hydrolysed. The standard tests showed that the enzyme in all the digests was active after the last determinations of reducing power had been made.

Table 2. *The hydrolysis of maltotetraose by the bacterial amylases*

(The digests were incubated at 35° at pH values of 4·8 for the clostridial enzyme and 6·0 for the streptococcal enzyme, and contained maltotetraose (14 mg.), 0·2M-sodium acetate (3 ml.), enzyme (66 units), water to 14 ml.)

Source of enzyme	Time of incubation	Apparent conversion of tetraose to maltose (%)
Clostridium butyricum	2 min.	37·1
	4·25 hr.	59·3
	20 hr.	81·5
A rumen *Streptococcus*	2 min.	46·5
	21 hr.	80·3
	45·5 hr.	91·9
	94·5 hr.	95·6

DISCUSSION

The simultaneous action on amylose and β-dextrin of the two enzymes described in this paper (Fig. 1), together with the results obtained by a comparison of the change in iodine stain and the apparent conversion to maltose of amylose, indicate that the enzymes are of the α-amylase type. With both enzymes the iodine stain of the polysaccharide changes from blue, through purple and red and then disappears during the hydrolysis of amylose or starch, a result which is given by α- and not by β-amylases. The two enzymes differ in the extent of conversion of amylose to sugars, during the initial stages of the hydrolysis. The enzyme of the rumen strain of *Cl. butyricum* shows a similarity to that of the enzyme from the pig caecum strain examined by Whelan & Nasr (1951), but differs from the streptococcal enzyme, which is similar to that of salivary α-amylase. This difference between the enzymes could be explained by assuming that the streptococcal and salivary α-amylases make a more endwise attack on the amylose chain splitting off low molecular weight sugars with a high reducing power and leaving dextrins of long chain length and high blue value. The clostridial enzymes, on the other hand, rapidly degrade amylose in a more random manner forming dextrins of short chain length and low blue value. Thus at any given apparent conversion to maltose the clostridial amylase digests have a lower EV (680 mμ.) than the salivary and streptococcal amylase digests. Further work to identify some of these intermediate products is in progress. This difference between the enzymes is maintained up to the end of the hydrolysis, the clostridial enzymes giving lower conversion limits than the salivary and streptococcal enzymes. On comparison of the extents of conversion of amylose to reducing sugars there is also, however, a difference between the amylases of the two strains of *Cl. butyricum*. The amylase from the pig caecum strain of organism hydrolysed starch and amylose to 65–66% apparent maltose, a figure which was not increased on prolonged incubation or addition of more enzyme. The amylase from the sheep rumen strain of *Cl. butyricum* rapidly hydrolyses amylose to about 80% apparent maltose and this extent of conversion slowly increases on prolonged incubation (Fig. 5). Starch and amylopectin are hydrolysed to smaller extents. The streptococcal

enzyme, however, again appears to be similar to salivary α-amylase in the final apparent conversion to maltose. Roberts & Whelan (1951) have recently reported a final conversion of amylose to 90% apparent maltose by salivary α-amylase. With the streptococcal enzyme also starch and amylopectin have lower extents of conversion to sugars than amylose. This is most likely due to the presence of α-1:6-linkages in amylopectin which are not hydrolysed by the α-amylases and which act as obstructions to the enzymes and lead to the formation of dextrins of molecular weight greater than maltose and maltotriose; these dextrins are then slowly hydrolysed. This is in accordance with the results of other workers (Meyer & Gonon, 1951; Myrbäck, 1950) who concluded that α-amylases do not hydrolyse α-1:6-linkages.

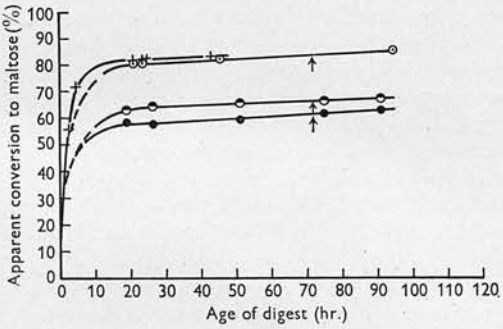

Fig. 5. The hydrolysis of starch fractions by the clostridial amylase at 35°. Digests at pH 4·8; amylose (⊙), starch (◓), amylopectin (●). Digest at pH 6·0; Amylose (+). Addition of enzyme to bring the activity to the initial value (↑).

In a later publication it is hoped to describe the isolation of the end products of the hydrolysis of amylose and starch by these enzymes, and a preliminary fractionation has shown that the clostridial enzyme produces from amylose a mixture of sugars of which maltose and maltotriose are the main constituents, together with a little glucose and maltotetraose. The end products of the action of the streptococcal enzyme on amylose were shown by paper chromatography to contain glucose, maltose and maltotriose. As mentioned above, neither enzyme hydrolyses maltose or maltotriose, so the slow increase in reducing power on prolonged incubation of the digests containing starch and starch fractions is most likely due to the degradation of remaining traces of maltotetraose, which is hydrolysed by the enzymes (Table 2), and other dextrins of slightly longer chain length. Whelan & Nasr (1951) reported that a preliminary investigation of the products of hydrolysis of amylose by the pig caecum clostridial amylase showed the presence of maltose and maltotriose, and later Roberts & Whelan (1951) isolated the end products of hydrolysis of amylose by salivary α-amylase and showed these, again, to be maltose and maltotriose. It would thus appear that maltotriose is a main product of the degradation of amylose by all these amylases. The difference in the final reducing powers of the digests containing amylose would then be mainly due to differences in the proportions of maltose, maltotriose and glucose present. In the products of hydrolysis of amylose these enzymes differ from the malt α-amylase and pig pancreatic amylase studied by Meyer & Gonon (1951) which are stated to give maltose and glucose as the end products of hydrolysis of amylose, maltotriose being a comparatively slowly hydrolysed intermediate product.

In further experiments it was found that the two organisms grew on media containing glucose,

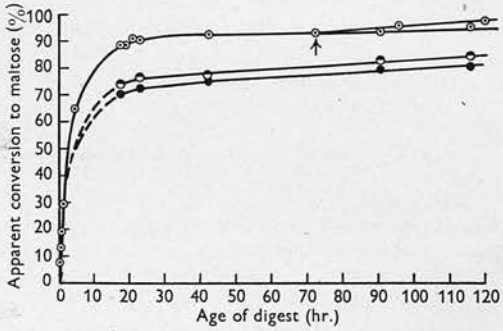

Fig. 6. The hydrolysis of starch fractions by the streptococcal amylase at 35°. Digests at pH 6·0; amylose (⊙), starch (◓), amylopectin (●). Addition of enzyme to bring the activity to the initial value (↑).

maltose or maltotriose as the only carbohydrates and that acids were produced by fermentation of these sugars. Thus these organisms can use as energy sources the main products of the hydrolysis of starch by their isolated extracellular amylases.

SUMMARY

1. Starch-degrading enzymes have been isolated in the form of stable freeze-dried powders from cell-free filtrates of cultures of sheep rumen strains of *Clostridium butyricum* and a *Streptococcus* grown in media containing dissolved starch. Their properties have been examined.

2. The enzymes belong to the group of α-amylases. The hydrolysis of starch and starch fractions by the enzymes has been investigated and the two enzymes have been shown to differ in the extents of conversion of these substrates to reducing sugars. The branch linkages in amylopectin act as obstructions to both enzymes.

3. The presence in the enzyme preparations of β-amylase, maltase, invertase, phosphatase or Q-

and R-enzymes could not be detected, but small traces of phosphorylase were present.

4. Maltose and maltotriose with some glucose are the main end products of the hydrolysis of amylose by these enzymes. Neither amylase hydrolyses these sugars, but maltotetraose is degraded by both enzymes.

5. A simple method for determination of α-amylase activity is described.

The authors wish to thank Dr W. J. Whelan of the University College of North Wales for helpful advice, and Prof. S. Peat, F.R.S., for permission to commence this work in the Chemistry Department, University College, Bangor.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 358.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Bailey, M., Whelan, W. J. & Peat, S. (1950). *J. chem. Soc.* p. 3692.
Baker, F., Nasr, H., Morrice, F. & Bruce, J. (1951). *J. Path. Bact.* **62**, 617.
Bourne, E. J., Haworth, N., Macey, A. & Peat, S. (1948). *J. chem. Soc.* p. 924.
Di Carlo, F. J. & Redfern, S. (1947*a*). *Arch. Biochem.* **15**, 333.
Di Carlo, F. J. & Redfern, S. (1947*b*). *Arch. Biochem.* **15**, 343.
Di Carlo, F. J. & Redfern, S. (1948). *Arch. Biochem.* **17**, 1.
French, D. & Knapp, D. W. (1950). *J. biol. Chem.* **187**, 463.
Green, D. E. & Stumpf, P. K. (1942). *J. biol. Chem.* **142**, 355.
Hobson, P. N., Pirt, S. J., Whelan, W. J. & Peat, S. (1951). *J. chem. Soc.* p. 801.
Hobson, P. N., Whelan, W. J. & Peat, S. (1950). *J. chem. Soc.* p. 3566.
Hobson, P. N., Whelan, W. J. & Peat, S. (1951). *J. chem. Soc.* p. 1451.
Hockenhull, D. J. D. & Herbert, D. (1945). *Biochem. J.* **39**, 102.
Hopkins, R. H., Dolby, D. E. & Stopher, E. G. (1942). *J. Inst. Brew.* **48**, 174.
Hopkins, R. H. & Kulka, D. (1942). *J. Inst. Brew.* **48**, 170.
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
Kerr, R. W., Cleveland, F. C. & Katzbeck, W. J. (1951). *J. Amer. chem. Soc.* **73**, 3916.
Masson, M. (1951). *Brit. J. Nutrit.* **4**, viii.
Meyer, K. H., Fuld, M. & Bernfeld, P. (1947). *Experientia*, **3**, 411.
Meyer, K. H. & Gonon, W. F. (1951). *Helv. chim. Acta*, **34**, 308.
Myrbäck, K. (1950). *Ark. Kemi*, **2**, 417.
Peat, S., Pirt, S. J. & Whelan, W. J. (1952). *J. chem. Soc.* p. 705.
Peat, S., Thomas, G. J. & Whelan, W. J. (1952). *J. chem. Soc.* p. 722.
Philips, L. L. & Caldwell, M. L. (1951*a*). *J. Amer. chem. Soc.* **73**, 3559.
Philips, L. L. & Caldwell, M. L. (1951*b*). *J. Amer. chem. Soc.* **73**, 3563.
Roberts, P. & Whelan, W. J. (1951). *Biochem. J.* **49**, lvi.
Rose, D. (1948). *Arch. Biochem.* **16**, 349.
Somogyi, M. (1945*a*). *J. biol. Chem.* **160**, 61.
Somogyi, M. (1945*b*). *J. biol. Chem.* **160**, 69.
Whelan, W. J. & Nasr, H. (1951). *Biochem. J.* **48**, 416.
Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.

Reprinted from
The Journal of Comparative Pathology and Therapeutics,
1952, Vol. 62, No. 4.

FERMENTATIVE ACTIVITIES OF SOME MEMBERS OF THE NORMAL COCCAL FLORA OF THE HORSE'S LARGE INTESTINE

F. ALEXANDER, MARGARET J. D. MACPHERSON AND A. E. OXFORD
The Rowett Research Institute, Bucksburn, Aberdeenshire.

Printed by C. Tinling & Co. Ltd., Liverpool, London and Prescot
and published by the University Press of Liverpool.

Reprinted from
The Journal of Comparative Pathology and Therapeutics,
1952, Vol. 62, No. 4.

FERMENTATIVE ACTIVITIES OF SOME MEMBERS OF THE NORMAL COCCAL FLORA OF THE HORSE'S LARGE INTESTINE

By

F. ALEXANDER, MARGARET J. D. MACPHERSON AND A. E. OXFORD

The Rowett Research Institute, Bucksburn, Aberdeenshire.

INTRODUCTION

The high incidence of diseases of digestion in the horse has long been recognised. Dobberstein and Dinter (1941) found that of 1121 autopsies on horses they accounted for 49·4 per cent. In view of this fact it is surprising that fundamental information on the physiology of digestion in the horse is conspicuously lacking. One reason for this has been the surgical problems involved in preparing fistulæ into the various parts of the bowel. However, some of these problems have already been surmounted. Alexander and Donald (1949) described a technique for the fistulation of the cæcum and large colon, and more recently Alexander (1952) has found it possible to prepare a horse with fistulæ in the cæcum and dorsal and ventral colons. By means of such fistulated animals observations on the fermentative processes of the large bowel have been made, and some of the more active bacteria involved have been isolated.

Particular attention has been paid to cocci in view of the predominantly coccal microflora of other alimentary sites, such as the rumen of sheep and cattle (Hungate, 1950; Sijpesteijn, 1951), where active carbohydrate fermentation is proceeding. It was hoped also to throw some light on the anomalous behaviour of *Streptococcus equinus* (Andrewes and Horder, 1906) the characteristic streptococcus of horsedung, which ferments but few sugars when compared with other fæcal streptococci (Orla-Jensen, 1943). By standard enrichment procedures several facultatively anærobic streptococcal strains of wide fermentative powers have been isolated from colon sites in fistulated horses and also a Gram-negative coccus. which is very active in fermenting lactate, a normal fermentation product of fæcal streptococci. This paper deals in detail with the fermentative behaviour of these bacteria and with the question of their identification.

METHODS

Experimental animals. Three ponies were used, two (B and G) having fistulæ in the right dorsal and ventral colons (hereinafter referred to as colons 4 and 1 respectively) and the third (T) fistulated in colon 1 only. The animals were maintained on hay and oats throughout.

Enrichment of bacteria in the colon contents. Most attention was paid to the fermentation of lactose, and of cellobiose a possible

intermediate in cellulose degradation, which are not usually attacked by *Str. equinus*. Material from the colon was withdrawn in the morning, collected in warm and chemically clean beakers, taken immediately to the 39° incubator and strained through 4 layers of clean, sterile surgical gauze in order to remove the greater part of the plant debris. The filtrate was quickly distributed in 20 ml. lots, into sterile 6″ test-tubes, each containing not more than 200 mg. of the solid, finely powdered sugar to be fermented previously sterilised by steam. After thorough mixing to dissolve the sugar, each test-tube was fitted with a clean and steam-sterilised cork bearing a bunsen-valve attachment, and all, save the unfermented controls which were immediately put in the cold room, were then incubated for 6 to 7 hours, when the fermentation was stopped by cooling to 0°. It was considered advisable not to continue the fermentations for a longer period in order to avoid an appreciable growth of chance contaminants.

Analysis of fermentation liquors. The pH as determined by the glass electrode was a sufficient indication of a good fermentation. It was invariably found that at values below pH 5.0 most of the sugar had disappeared. Lactic acid was determined colorimetrically (Hillig, 1942) and the total volatile fatty acid by repeated steam distillation after acidification. In some instances a viable bacterial count was made, both before and after fermentation in 1 per cent Bacto Yeast Extract agar medium containing 1 per cent lactose or cellobiose, by the roll-tube method. The roll-tubes were incubated for 2 days at 39° in a N_2+5 per cent CO_2 atmosphere. Colonies which were almost invariably cocci were then picked off for microscopic examination and purification by replating in the usual way.

Isolation of lactate-fermenting bacteria. A highly-diluted colon liquor was judged to contain lactate-decomposing bacteria if marked disruption occurred in shake agar tubes after 24 hours incubation. The medium was composed of Na lactate, 2 per cent; Bacto Yeast Extract, 0·67 per cent; Bactopeptone, 0·33 per cent; K_2HPO_4, 0·33 per cent; KH_2PO_4 0·1 per cent; agar 2 per cent and sodium lactate as the source of carbohydrate. The initial pH was between 6 and 7. A control with no lactate in the medium never showed gas production even after several days' incubation.

For the isolation of the lactate fermenting bacteria, sterile Pasteur pipettes were inserted into the bottom of the disrupted shake cultures, where there was usually a free space containing some turbid liquid, and a few drops withdrawn for microscopic examination. If found to be rich in Gram-negative cocci, the rest of the liquid was transferred to a second shake culture from which blood agar plates incubated anærobically at 40° were prepared. Purification was carried out by alternate culture of isolated colonies in liquid lactate medium, followed by replating on blood agar. When only Gram-negative diplococci were seen in stained

films and the final isolate also disrupted lactate agar, it was concluded that a pure culture had been obtained.

Serological methods used for identification. Precipitin tests were carried out with extracts made both by the acid method of Lancefield (1933) and by formamide extraction as described by Fuller (1938). The following group specific antisera were available: A, B, C, D, E, F, G, H, and K from Burroughs Wellcome & Co., and N from Dr. Mattick, Shinfield, Reading. Most useful information was obtained by the application of the Neufeld 'capsular-swelling' reaction used by Mirick, Thomas, Curnen and Horsfall (1944) for *Str. salivarius* and related strains.

Antisera homologous to the newly isolated horse streptococci were prepared by the method of Lancefield (1934). Cultures for injection into rabbits were prepared by re-suspending cells from 18 hour broth cultures in 0·85 per cent NaCl, containing 0·2 per cent formalin, to a volume equal to 5 per cent of that of the original culture. After 48 hours at 0° the bacterial suspensions were sterile. Immediately before use suspensions were diluted 20 times with saline. Doses of 1 ml. were injected intravenously into rabbits daily for 1 week, followed by a week's rest. Three courses of injections were given.

RESULTS

Properties of Colon 1 and Colon 4 Liquors

The respective micro-fauna of these liquors are quite distinct and serve to differentiate between them (Strelkow, 1939; Adam, 1951). There are also differences in chemical and physical properties. Strained colon 4 liquor is in general thicker and darker in colour than colon 1 liquor, and contains rather more material precipitable by dilute acetic acid (mucoprotein?). Both are essentially phosphate buffers (equivalent to about 0.15 per cent K_2HPO_4) and are neutral in reaction. Colon 1 liquor also appears to contain a little bicarbonate, since its pH rises to 8·0-8·5 on standing. In each instance the buffering capacity is heightened by the pressure of appreciable concentrations of volatile fatty acid anions.

Sugars other than lactose fermented by colon contents

Pentoses were not readily attacked. Cellobiose, fructose, glucose, maltose, starch and sucrose were utilised by both colon 1 and colon 4 liquors, e.g. 0·25 per cent of dissolved substrate disappeared in 6 hours. Galactose and lactose were more readily attacked by colon 1 than by colon 4 liquor.

Lactose fermentations by colon 1 and colon 4 liquors

In these experiments the colon 1 contents were from Pony T and the colon 4 contents from Pony B. The results of two separate 7 hour fermentation tests are summarised in Table I. Isolations of cultures were made from the colonies developing in the viable

counts underlined in Table I with results summarised in Table II. The seven streptococcal isolates denoted by asterisks in that table were kept in cultivation and a more extended study made of their fermentation reactions. Three of these strains were kept for the serological study recorded later.

TABLE I

RESULTS OF FERMENTATION OF LACTOSE AND OTHER CARBOHYDRATES BY COLON LIQUORS 1 AND 4.

Experiment	*Carbohydrate*	*Final pH*		*Viable bacterial count on lactose-yeast extract-agar (per ml ; × 10⁶)*	
		Colon 1	*Colon 4*	*Colon 1*	*Colon 4*
I	None (after incubation)	7·0	No test	No test	1
	Lactose	4·85	5·05	*68·75*	*420*
	Cellobiose	4·7	4·6	150*	87·5*
	Mannitol	6·7	6·7	No test	No test
II	None (before incubation)	6·6	7·2	*2*	*2·5*
	None (after incubation) ...	7·0	6·8	No test	No test
	Lactose	4·7 ; 4·7	5·7 ; 5·8	*90*	*15*
	Cellobiose	4·6 ; 4·5	4·9 ; 4·8	No test	164·5*

*On cellobiose-yeast-extract agar.

TABLE II

NUMBER OF LACTOSE AND NON-LACTOSE FERMENTING STREPTOCOCCI FROM ISOLATED COLONIES.

Source of streptococci and kind of fermentation liquid examined	*No. of colonies picked off*	*No. of lactose-fermenting strains isolated*
Colon 1 ; lactose	5	5
Colon 4 ; lactose	9	7
Colon 1 ; before fermentation	7	6*
Colon 1 ; lactose	1	1*
Colon 4 ; before fermentation	2	0
Colon 4 ; lactose	3	0

* Kept in subculture.

Properties of the lactose-fermenting streptococci. These streptococci were short-chained or diplococcal in appearance, the chains being embedded in a thin mucoid material, particularly on solid media. The strains were non-haemolytic and only one strain which quickly died out in sub-culture, fermented mannitol. The others fermented arabinose, glucose, galactose, maltose, lactose, trehalose,

raffinose, starch, dextrin and æsculin with production of acid, chiefly *d-lactic*. After 5 days' incubation the pH which was initially 7·6 had fallen to between 4 and 5. The following were not fermented: xylose, rhamnose, glycerol, mannitol, dulcitol, sorbitol, inositol. One strain (J) fermented sucrose, one strain (A) fermented both inulin and salicin. The two strains (A. J) and a third (N) which gave typical fermentation reactions, were positive to the methyl red test, did not liquefy gelatin, hydrolysed Na hippurate and reduced methylene blue with clot in skim milk medium.

Antigenic relationships of the lactose-fermenting streptococci. Antigenic extracts prepared from strains A, J and N by the formamide method of Fuller (1938) gave positive precipitin reactions with homologous antiserum prepared against strain J. On the other hand they gave entirely negative results when tested against all commercial sera, or against antisera from sheep's rumen streptococci (MacPherson, 1952). These results were most convincingly confirmed by means of the Neufeld capsular swelling reaction. Only in the presence of the homologous antiserum was the capsule clearly outlined as a greyish zone surrounding each pair or short chain of cocci. The addition of antiserum J to the colon liquor itself revealed definite capsulation in a small proportion of the cocci present. With normal serum no such capsulation could be demonstrated (Figs. 1 and 2).

FIG. 1

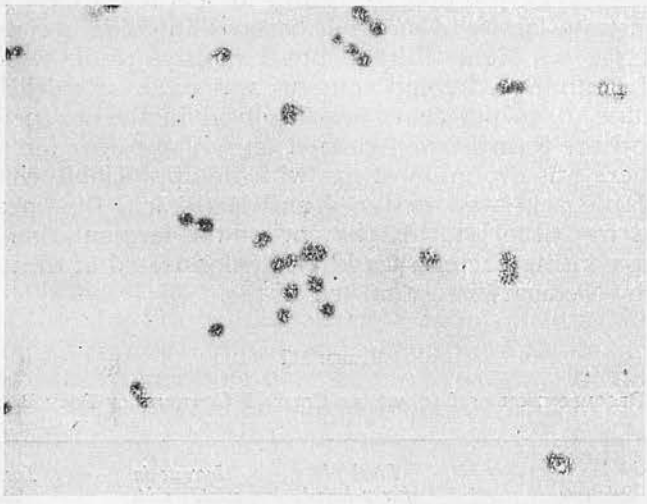

FIG. 1. Horse colon streptococcus strain J and normal rabbit serum. No obvious capsule. × 2500

Lactate fermentations by colon 1 and colon 4 liquors

On all occasions with all 3 ponies disruption of the agar occurred when the colon 1 contents were diluted 600 times with

FIG. 2

FIG. 2. Streptococcus J and homologous antiserum, showing clear delineation of the capsule. × 2500

Both were wet preparations stained with methylene blue.

sterile water before testing. On some occasions dilutions of 10,000 or even 25,000 gave a positive result. It was not always possible to demonstrate lactate-fermenting bacteria in colon 4 contents if the material was highly diluted but a positive result was always obtained if undiluted colon contents was used. If sterile Na or NH_4 lactate (0·35 per cent) was dissolved in the strained colon liquor and the fermentation carried on without agar for 7 hours at 39° there was evolution of gas with no appreciable change in pH. Volatile acid was produced and lactic acid disappeared as indicated in Table III. At the end of the fermentation period lactate-fermenting bacteria could be demonstrated at dilutions of 100,000 by the agar-disruption technique.

TABLE III

DECOMPOSITION OF LACTATE BY COLON 11 CONTENTS OF PONY B

Substrate	*Final pH after 7 hours' fermentation*	*Increase in volatile acid, calc. as acetic mg%*	*Loss of lactic acid mg%*
None	6·41	316	None
Na lactate	6·68	2216	1530
NH_4 lactate	6·62	1998	2220
Free lactic acid	6·62	1114	1530

Properties of the lactate-fermenting Gram-negative cocci. These organisms were strict anærobes with rather exacting growth requirements, blood agar or cooked meat medium being necessary for their continued propagation. No carbohydrates were fermented and among all the hydroxy and dicarboxylic acids tried as substrates, gas was produced only from lactate, and to a lesser extent from tartrate. The colonial appearance on agar and most other properties were exactly as described in detail by Johns (1951) for the organism obtained from the sheep's rumen, and presumed to be *Veillonella gazogenes.* In view of this and of the frequent occurrence of this coccus in intestinal sites no further investigation of our equine strain was considered necessary.

DISCUSSION

It is clear that the *Str. equinus* of fæcal origin, which ferments only a few carbohydrates, is by no means the only streptococcus to be found in the colon of the horse. The streptococci which we have isolated, in so far as fermentation reactions are concerned, resemble the well-known *Str. bovis* which has wide fermentative powers (Orla-Jensen, 1943), but on serological grounds seem to fall into a group of their own. They are capsulated and their presence, either in pure or in mixed cultures or in the colon fluids, can conveniently be demonstrated by means of the Neufeld capsular swelling reaction provided homologous antiserum is used. We are of the opinion that this very simple serological test might be more widely used in the identification of encapsulated streptococci in the natural situations in which they occur. A further study (MacPherson, 1952) along these lines is in progress with other animals.

The chief fermentation product of these colon streptococci is lactic acid which is not usually found in colon liquor. Consequently, the presence in the colon of the ubiquitous *Veillonella gazogenes,* which ferments no sugars but only lactate to volatile fatty acids is not unexpected. Since a more vigorous fermentative activity is observed in the ventral than in the dorsal part of the colon, the greater concentration of the Gram-negative coccus in the former site and the presence of bicarbonate ions in quantity in colon 1 liquor only is readily explained. It seems unlikely, however, that *V. gazogenes* can be responsible for the production of the greater part of the high volatile fatty acid content which has been estimated at 0·3 to 0·7 per cent of colon liquor (Alexander, 1952), partly because it seems to be present in insufficient numbers and partly because the colon does not normally contain large volumes of gas. The fermentation of lactate by *V. gazogenes* would undoubtedly produce hydrogen as well as CO_2 (Johns, 1951). It may be that lactate is very rapidly absorbed from the colon and only small amounts remain for fermentation with *V. gazogenes.* The fermentative and absorptive processes of the horse's colon evidently

need a much fuller study, which one of us (F.A.) hopes to carry out in the near future.

A point of some interest is the fact that the common streptococcus of horse dung differs so markedly from the organisms isolated from the colon. It is possible that selective feeding on the part of ciliate protozoa, which are present in large numbers in colon 4 (Adam, 1951), may account for this anomaly.

SUMMARY

Streptococci capable of fermenting lactose and starch have been isolated from the large colon contents of fistulated ponies.

They form a capsule which is easily demonstrated by the capsular swelling reaction described by Neufeld.

They can be differentiated serologically from other types of streptococci.

The colon also contains the Gram-negative coccus *Veillonella gazogenes,* an anærobic fermenter of lactate.

REFERENCES

Adam, K. M. G. (1951). *Parasitol.*, **41,** 301.
Alexander, F. (1952). *In preparation.*
Alexander, F., and Donald, D. E. (1949). *J. comp. Path.*, **59,** 127.
Andrewes, F., and Horder, T. (1906). *Lancet, ii,* 708, 775, 852.
Dobberstein, J., and Dinter, W. (1941). *Arch. wiss, prakt. Tierhielk.*, **76,** 256.
Fuller, A. T. (1938). *Brit. J. exp. Path.*, **19,** 130.
Hillig, F. (1942). *J. Assoc. official agric. Chem.*, **25,** 602.
Hungate, R. E. (1950). *Bact. Rev.*, **14,** 1.
Johns, A. T. (1951). *J. gen. Microbiol.*, **5,** 317.
Lancefield, R. C. (1933). As quoted in Mackie & McCartneys' *Handbook of Practical Bacteriology*, 8th Edition, pp. 320-323.
Lancefield, R. C. (1934). *J. exp. Med.*, **59,** 441.
MacPherson, M. J. D. (1952). *In preparation.*
Mirick, G. S., Thomas, L., Curnen, E. C., and Horsfall, F. L. (1944). *J. exp. Med.*, **80,** 431.
Orla-Jensen, S. (1943). *Die echten Milchsäurebakterien.* Copenhagen; Ejhar Munksgaard.
Sijpesdteijn, A. K. (1951). *J. gen. Microbiol.*, **5,** 869.
Strelkow, A. (1939). *Uchenyeie Zapiski pedagogich, instituta im. Gercena,* **17,** p. 262. (Quoted by Adam, K. M. G. (1951). *Parasitol,* **41,** 301.)

[*Received for publication, March 10th,* 1952.]